Tânia Cristina da Luz Palma

DEGRADATION STUDIES OF WIDELY USED PHARMACEUTICAL COMPOUNDS BY HETEROGENEOUS PHOTOCATALYSIS AND BACTERIAL COMMUNITIES AND ISOLATES

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“Degradation studies of widely used pharmaceutical compounds by heterogeneous photocatalysis and bacterial communities and isolates”

Declaração de Autoria do Trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Tânia Cristina da Luz Palma

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I dedicate this thesis
to my parents and grandparents,
to Miriam and Morgana

Eu dedico esta tese
aos meus pais e avós,
à Miriam e
à Morgana

"You are what your deep, driving desire is.
As your desire is, so is your will.
As your will is, so is your deed.
As your deed is, so is your destiny."

Brihadaranyaka Upanishad

List of Publications

This thesis is based on the following original articles:

Palma, T., Monteiro, O. C., Pinto da Costa, J. P., Lourenço, J. P., Costa, M. C. (2013). Production of PbS (galena) nanoparticles and nanocomposites using biologically generated sulphide. Proceedings of the 2nd International Conference of Wastes Solutions, Treatments and Opportunities (WASTES, Braga, Portugal, 2013). ISSN 2183-0568. – (CHAPTER 2)

Palma, T. L., Vieira, B., Nunes, J., Lourenço, J. P., Monteiro, O. C., Costa, M. C. (2020). Photodegradation of chloramphenicol and paracetamol using PbS/TiO_2 nanocomposites produced by green synthesis. Journal of the Iranian Chemical Society. https://doi.org/10.1007/s13738-020-01906-1 – (CHAPTER 2)

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Palma, T. L., Martins, A., Palma, R., Páscoa, A., Ennis, N., Carlier, J. D., Costa, M. C. (2019). Assembly, start-up and optimization attempt of an aerobic granular sludge SBR system. (*in preparation*) – (CHAPTER 8)

Abstract

The present thesis was focused on degradation studies of five extensively and globally used pharmaceutical compounds: chloramphenicol, paracetamol, fluoxetine and 17α-ethynilestradiol which are considered emerging pollutants that enter in the environment due to its inefficient removal by the conventional wastewater treatments plants. Photodegradation and biodegradation assays were realized. The photodegradation of chloramphenicol and paracetamol was successfully performed by heterogeneous photocatalysis using PbS/TiO_2 nanocomposites, as catalysts, which were synthesized using biological sulphide produced by sulphate reducing bacteria in batch cultures. Although photodegradation demonstrated to be a faster process, biodegradation is preferable since it occurs without the use of external compounds, such as catalysts, using only the microorganisms already present in the environment. Communities and bacterial isolates with ability to remove and use these drugs as unique carbon sources were obtained using sludge collected from an oxidation ditch and a lagoon system of WWTPs. An initial aerobic bacterial community suffered a shift to favor the genera *Pseudomonas sp*; *Flavobacterium*, *Dokdonella* and *Methylophilus* which displayed high efficiency in removing 50 mg/L paracetamol as sole carbon source and are described for the first time as paracetamol degraders. Bacteria capable of effectively biodegrade paracetamol were isolated in aerobic conditions, suggesting for the first time that members of "*Bacillus cereus group*", *[Brevibacterium] frigoritolerans*, *Corynebacterium nuruki* and *Enterococcus faecium* can use paracetamol as carbon source, whereas the isolates obtained for fluoxetine and 17α-ethynilestradiol only partially removed these drugs. An anaerobic consortium which was able to degrade 20 mg/L of FLX, as unique carbon source, to values below the limit of detection was obtained. The final consortium was mainly composed by genera *vadinBC27* wastewater-sludge group, *Macellibacteroidetes*, *Dethiosulfovibrio*, *Bacteroides*, *Tolumonas*, *Sulfuricurvum*, *f_Enterobacteriaceae*_OTU_18, which are for the first time assumed as FLX biodegraders. In the biodegradation of 20 mg/L FLX, a possible metabolite, 2,3-dimethyl-5-(trifluoromethyl) benzene-1,4-diol, was detected after 28 of assay. Several attempts to optimize an aerobic granular system were performed to degrade ibuprofen. This process is still ongoing.

Keywords: Heterogeneous photocatalysis; Biodegradation; Bacterial communities; Isolates; Pharmaceuticals; Secondary metabolites

Resumo

A presente tese foca-se em estudos de degradação de 5 fármacos considerados poluentes emergentes, são estes: cloranfenicol, paracetamol, fluoxetina, 17α-etinilestradiol e ibuprofeno. Estes são considerados potenciais ameaças aos ecossistemas ambientais, à saúde e à segurança humana, especialmente devido ao seu uso extensivo a nível mundial. Os tratamentos de águas residuais convencionais têm-se mostrado ineficientes na remoção total destes fármacos resultando na sua libertação para o ambiente. Atualmente, uma das maiores preocupações é a contaminação incessante dos ambientes aquáticos com estes poluentes e os problemas ecológicos inerentes. Com o objetivo de conhecer, desenvolver ou melhorar os processos já existentes, foram realizados estudos de fotodegradação e biodegradação visando encontrar novas alternativas que sejam baratas e simples e que permitam uma completa remoção destes fármacos nas estações de tratamento de águas residuais (ETARs). Nos estudos de fotodegradação foram sintetizados com sucesso nanocompósitos de PbS/TiO_2 (fotocatalisador heterogéneo) utilizando sulfureto produzido biologicamente pelas bactérias sulfato redutoras (BSR) em batch. A importância deste processo está no aproveitamento de resíduos tóxicos, como o sulfureto e iões chumbo em solução aquosa, para formulação de um catalisador eficiente na remoção destes poluentes (fármacos). Estes nanocompósitos, foram empregues com êxito como catalisadores na remoção de cloranfenicol e paracetamol, revelando uma elevada eficiência de remoção (>93%), após 240 minutos de irradiação. Nos ensaios de biodegradação obtiveram-se comunidades e isolados bacterianos com capacidade de utilizar os fármacos em estudo, como única fonte de carbono e que são descritos pela primeira vez como tendo um papel muito importante na remoção destes poluentes. Identificou-se uma comunidade bacteriana aeróbia que apresentou elevada eficácia na remoção de paracetamol como única fonte de carbono (97 ± 3%). Ao longo do ensaio verificou-se um "shift" na população inicial levando a um favorecimento dos géneros *Pseudomonas sp*; *Flavobacterium*, *Dokdonella* e *Methylophilus*, revelando pela primeira vez o seu possível envolvimento na degradação do paracetamol. Os metabolitos identificados nesse processo foram 4-aminofenol, hidroquinona e um composto desconhecido. Isolaram-se bactérias com capacidade de biodegradar paracetamol utilizando como inóculo as lamas recolhidas numa vala de oxidação, sugerindo pela primeira vez que membros de "*Bacillus cereus* group" (associados *Bacillus pacificus* ou *Bacillus paranthracis*), [*Brevibacterium*] *frigoritolerans*,

Corynebacterium nuruki e *Enterococcus faecium* têm a capacidade de usar este fármaco como única fonte de carbono. Uma espécie pertencente ao "*Bacillus cereus* group" degradou 200 mg/L de paracetamol, abaixo dos níveis de deteção, sem formação aparente de metabolitos secundários tóxicos e removeu cerca de 95 ± 5% de 500 mg/L do fármaco, após 144 h de incubação a 28 ºC. Durante o processo de degradação do paracetamol foram detetados os metabolitos 4-aminofenol, hidroquinona e ácido 2-hexenóico. Realizaram-se estudos de biodegradação com uma comunidade bacteriana enriquecida a partir de uma amostra de lama recolhida num sistema de lagunagem em que foram favorecidas as BSR. As culturas foram efectuadas na presença de fluoxetina e lactato e usando o fármaco como única fonte de carbono, em condições redutoras e anaerobiose. O consórcio obtido revelou ter capacidade de degradar 20 mg/L e 50 mg/L de fluoxetina para valores abaixo do limite de deteção, após 28 e 31 dias, respetivamente. Utilizando fluoxetina como única fonte de carbono, verificou-se que o consórcio removeu 20 mg/L de fluoxetina abaixo dos limites de deteção e 66 ± 9% de 50 mg/L do fármaco, após 31 dias. Norfluoxetina foi identificada na degradação de 50 mg/L fluoxetina, enquanto na biodegradação de 20 mg/L de fluoxetina, foi detetado outro possível metabolito, o 2,3-dimetil-5-(trifluorometil)benzeno-1,4-diol, após 28 dias de ensaio. A população bacteriana inicial era constituída principalmente por *Desulfomicrobium* e *Desulfovibrio*, enquanto que no decurso das experiências usando fluoxetina como única fonte de carbono, verificou-se a ocorrência de um claro "shift" na comunidade com o aumento do grupo *Wastewater-sludge group vadinBC27*, *Macellibacteroidetes*, *Dethiosulfovibrio*, *Bacteroides*, *Tolumonas*, *Sulfuricurvum*, f_*Enterobacteriaceae*, bactérias identificadas pela primeira vez como possíveis degradadoras de fluoxetina. Em aerobiose, foram preparadas culturas inoculando o efluente bruto com a lama, simulando as condições da ETAR. Neste ensaio a remoção de fluoxetina foi de aproximadamente 89%, aparentemente com uma contribuição mínima de adsorção à lama. Durante este ensaio, a população inicial sofreu um "shift" com o aumento de estirpes dos géneros *Aeromonadales*_OTU_14, *Acinetobacter*, *Rheinheimera*, *Shewanella*, *Pseudomonas*, f_*Pseudomonadaceae*_OTU_4914; OTU_24; f_JI49D030_OTU_6171, *Methylobacillus* e *Piscinobacter*, sendo apontados pela primeira vez como os prováveis responsáveis pela biodegradação da fluoxetina. Da lama onde se obteve a comunidade anterior foram selecionados seis isolados com capacidade de utilizar fluoxetina como única fonte de carbono que foram identificados como *Pseudomonas putida*, *Enterobacter ludwigii*, *Pseudomonas nitritireducens*,

Alcaligenes faecalis, *Pseudomonas aeruginosa* e *Pseudomonas nitroreducens*. *Pseudomonas nitroreducens* foi a mais eficiente, com remoções de 55 ± 1% e 21 ± 1% a 20 mg/L e 50 mg/L de fluoxetina, respetivamente, após 168 h. Os isolados obtidos não apresentaram elevadas percentagens de remoção deste fármaco, contudo estas foram significativas quando comparadas com o controle negativo. Embora o principal mecanismo de remoção de fluoxetina descrito na literatura seja por adsorção, neste estudo a biodegradação parece desempenhar o papel principal na degradação do fármaco. Das lamas ativadas foram obtidos isolados bacterianos com capacidade de crescimento na presença de 50 mg/L de 17α-etinilestradiol a partir de uma lama recolhida na vala de oxidação da ETAR Noroeste de Faro, sendo estes identificadas como *Acinetobacter bouvetii*, *Acinetobacter kookii*, *Pantoea agglomerans* e *Shinella zoogloeoides*. Os resultados revelaram que *Acinetobacter bouvetii*, *Acinetobacter kookii*, *Pantoea agglomerans* e *Shinella zoogloeoides*, degradaram 47 ± 4%, 55 ± 3%, 64 ± 4% e 35 ± 4%, respetivamente de 13 mg/L 17α-etinilestradiol, após 168 h a 28 ºC. A degradação de 17α-etinilestradiol por estes isolados resultou em vários intermediários/vias possíveis que são mais facilmente degradados e menos tóxicos do que o composto original. Os compostos detetados foram: ácido manónico 1,4-lactona pertencente às estruturas "gama lactona", ácido 4-hidroxi-2-quinolinacarboxílico, ácido 2,3-di-hidroxibenzóico, ácido D-glucónico e ácido Z-aconítico, ácido 2-pentenodióico (ácido insaturado) e o ácido 2-butenodióico após possível meta-clivagem do anel A. O ácido 2-butenodióico (ácido fumárico), é um ácido dicarboxílico e um metabolito intermediário no ciclo dos ácidos tricarboxílicos/ciclo de Krebs. Em relação ao sistema granular realizaram-se diferentes abordagens a nível experimental visando optimizar o sistema e a degradação do ibuprofeno. Este processo ainda está em curso.

Palavras-chave: Fotocatálise Heterogénea; Biodegradação; Comunidades bacterianas; Isolados; Fármacos; Metabolitos secundários

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List of Abbreviations

ACN - acetonitrile
AGS - aerobic granular sludge
AGS-SBR - aerobic granular sludge-sequencing batch reactor
AMD - acid mine drainage
B.E.T. - Brunauer, Emmett and Teller
BTEX - benzene, toluene, ethylbenzene and xylene
CAP – chloramphenicol
CB - conduction band
CD – carbon dots
CFX - cefuroxime
COD - chemical oxygen demand
CPR – cefpirome
CTX - cefotaxime
CYPs - cytochrome P450 enzymes
DNA – deoxyribonucleic acid
dNTPs – deoxynucleotide
DBP - dibutyl phthalate
DO - dissolved oxygen
DP – diameter of the particle
DRS - diffuse reflectance spectroscopy
EBPR - enhanced biological phosphorus removal
EDCs - endocrine-disrupting chemicals
EDS - energy-dispersive X-ray system
EDTA - ethylenediamine tetraacetic acid
ELSD - evaporative light scattering detector
EPs - emerging pollutants
E1 – estrone
E2 – estradiol
E3 – estriol
EE2 - 17α-ethinylestradiol
E_h - redox potential

Flame-AAS - flame atomic absorption spectroscopy
FLX – fluoxetine
GCMS - gas chromatography-mass spectrometry
HPLC – high performance liquid chromatography
IBU – ibuprofen
IC_{50} - half maximal inhibitory concentration
Kapp – apparent rate constant
KM - Kubelka-Munk
LOD - limits of detection
LOQ - limit of quantification
MBR - membrane bioreactor
MSM - mineral salt medium
MWW – municipal wastewater
NAPQI - N-acetyl-p-benzoquinone imine
NCBI - national center for biotechnology information
ND - not detected
NFLX – norfluoxetine
NOEC - no observed effect concentration
NR - no removal
NSAIDs - nonsteroidal anti-inflammatory drug
NT - not tested
OD_{600nm} – optical density at 600 nm
OPs - organophosphorus pesticides
OUT - operational taxonomic unit
PAHs - polycyclic aromatic hydrocarbons
PANI - polyaniline
PAR – paracetamol
PCA - plate count agar
PPCPs - pharmaceuticals and personal care products
PCR - polymerase chain reaction
PEC – predicted environmental concentrations
PES – polyethersulfone
PHC - petroleum hydrocarbon
PMMA - poly(methyl methacrylate)
PMS - peroxymonosulfate
PP – polypropylene
RDP - ribosomal database project
rRNA - ribosomal ribonucleic acid
RT - retention time
SAED - selected area electron diffraction
SBRs - sequence batch reactors
SEM - scanning electron microscopy
SRA - SET and ring finger associated
SRB - sulphate-reducing bacteria
SSRIs - selective serotonin-reuptake inhibitors
SVT – sludge volume index
t - time
TAE - tris- 1% acetate-EDTA
TCA – tricarboxylic acid cycle
7- TFHOD - 2-hydroxy-6-oxo-7,7,7-trifluorohepta-2,4-dienoate hydrolase

TFM - 4-trifluoromethyl
TMA – trimethylamine
TMS - trimethylsilyl
TEM - transmission electron microscopy
TGM - thioglycolate medium
TSS - total suspended solids
UV – ultraviolet
VB - valence band
WWTP - wastewater treatment plant
XRD - X-ray powder diffraction

CHAPTER 1

General Introduction

1. GENERAL INTRODUCTION

1.1. Emerging pollutants an overview

Nowadays with the fast-technological advances and subsequent arise of new chemicals in which are included pharmaceutical drugs, personal care products and pesticides that are used systematically and worldwide, one of the biggest concerns is the incessant contamination of the aquatic environments by these pollutants and its inherent ecological issues.

These compounds are called emerging pollutants (EPs) since they are not covered by any water quality regulation but are considered potential threats to environmental ecosystems, human health and safety, especially because of their large-scale production and use, resulting in their release into the environment usually via sewage and/or wastewater treatment plant (WWTP) discharges (Deblonde *et al*. 2011; Gogoi *et al*. 2018).

Nevertheless, these organic compounds known to produce harmful effects are not only discharged in water sources but also present in landfills and agricultural resources (Bagheri and Mohseni, 2015; Sobhan *et al*. 2015; Victoria *et al*. 2015; Anandan *et al*. 2010).

Included in EPs there are the pharmaceutical drugs chloramphenicol, paracetamol, fluoxetine, 17α-ethinylestradiol and ibuprofen, whose degradation is the focus of the present thesis.

These drugs enter in WWTP by human excretion (urine and faeces) (Daughton and Ruhoy, 2009) and by inappropriate disposal of unused and expired drugs by flushing them through the toilet, going down to household drains. In addition to households, there are also other sources responsible for the entry of drugs into the environment, such as hospitals, agriculture, livestock and industries (**Figure 1.1**).

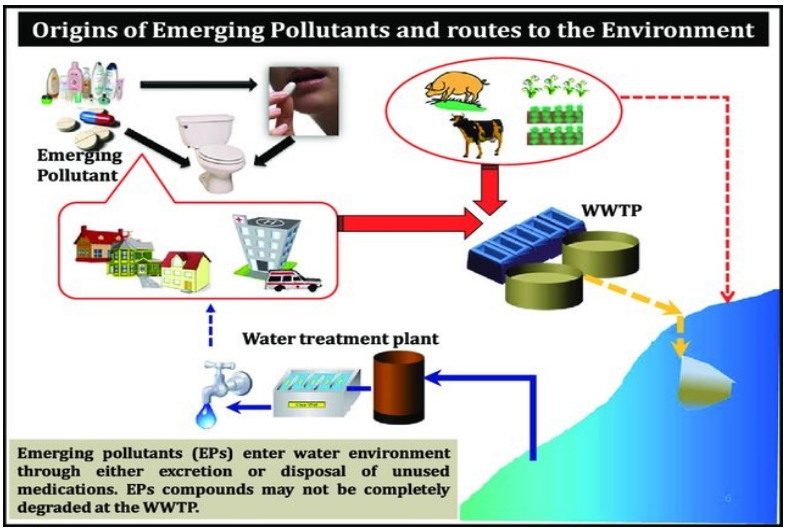


Figure 1.1 Conceptual depiction of the origin of emerging pollutants (EPs) and their route to the environment (adapted from Gogoi *et al*. 2018)

Hospital wastewaters contain high concentrations of pharmaceuticals (Gómez *et al*. 2006), however, due to the relatively small flow, the stream is generally diluted in the sewer system by a factor 100 before it enters the WWTPs (Kümmerer and Helmers, 1997). In addition, some hospitals have their own tailormade WWTP that aims the reduction of drug emissions in the sewage system (Le Corre *et al*. 2012; Wilt, 2018). The diluted wastewater coming from hospitals, discharged in the sewage system, origins that less than 15% of the total drugs load fed to WWTPs come from that source (Le Corre *et al*. 2012; Wilt, 2018). Also, the excess of antibiotics and hormones in the livestock feed enter in the water cycle by waste lagoons (Fent and Weston, 2006; Kümmerer, 2009; Gogoi *et al*. 2018).

Conventional WWTPs are typically designed for the removal of bulk organic matter, nitrogen and phosphorus but not for drugs removal. Although some drugs are removed during wastewater treatment, several of them are inefficiently removed by the treatment processes. Also, if the income quantity of some drugs overwhelms the treatment capacity, they are discharged in the WWTP effluent (Deblonde *et al*. 2011; Verlicchi *et al*. 2012). These issues lead that contamination occurs, thus any form of those untreated hazardous pollutants disposal or discharge into water sources, land, or air causes or may cause acute (short-term) or chronic (long-term) injury to the Earth's ecological balance or diminishes the quality of life of all the living beings (Chong *et al*. 2010; Pelaez *et al*. 2012; Schwarzenbach *et al*. 2010; Saravanan *et al*. 2017).

Concerning biodegradation, behaviour and fate of EPs in aquatic systems, much remains to be discovered, often leading to simplified assumptions underestimating the

real environmental risk assessments. Additionally, they may have an impact on wastewater treatment processes by increasing or decreasing sewage or industrial contaminant removal efficiency (Deblonde *et al*. 2011). Thus, the need to search for new, cost-effective and eco-friendly processes to detoxify the environment from these harmful EPs is of outmost importance.

Among the several processes that are used to degrade the pharmaceutical drugs, two of the most important ones, photodegradation by heterogeneous photocatalysis and biodegradation, belong to the category of eco-friendly and economic treatments that can be applied to WWTPs.

1.2. Remediation methods of polluted waters

1.2.1. Photodegradation by heterogeneous photocatalysis

Most of the conventional wastewater treatments direct the contamination from water into an alternative phase, requiring an additional treatment and/or disposal of the pollutants (Lee *et al*. 2015; Yongrui *et al*. 2015; Saravanan *et al*. 2017). Also, conventional biological treatments may not be adequate with nonbiodegradable and recalcitrant compounds (*e.g.* strong chemical oxidants, synthetic dyes), which can represent a potential threat to the nature (Lee *et al*. 2015; Yongrui *et al*. 2015; Saravanan *et al*. 2017).

Thus, among the novel treatment processes that can be used to degrade and detoxify the environment from EPs there is photocatalysis, a method that is gaining considerable attention in environmental remediation (Zeltner and Anderson, 1996). Photocatalytic decontamination of wastewater is a process that combines the use of a heterogeneous catalyst, such as semiconductors, and solar energy (Zhang *et al*. 1994).

Semiconductor photocatalysis, focused on TiO_2, has been applied to a variety of environmental issues of interest in addition to water and air purification (Hoffmann *et al*. 1995). In addition to TiO_2, several other types of photocatalysts are deeply studied and under use, such as ZnO and CdS, that are currently applied in deodorization, as antimicrobial, in air and water purification and in wastewater treatment, because of their attractive physical and chemical performances, low cost and availability with excellent stability (Fereshteh *et al*. 2014; Peter, 2015).

Thus, the application of semiconductors in photocatalysis for degrading undesirable organics dissolved in air or water is well documented and has been successful for a wide

diversity of compounds (Hoffmann *et al*. 1995). Several laboratories and field photocatalytic studies were performed in hazardous organic compounds such as alcohols, carboxylic acids, amines, herbicides and aldehydes, which demonstrated to be photocatalytically oxidized and ultimately mineralized to CO_2, H_2O, and/or other nontoxic products such as simple mineral acids (Ahmed and Ollis, 1984; Bagheri and Julkapli, 2015).

1.2.1.1. Semiconductors

A semiconductor is a material with conductivity between insulator and conductor (Belver *et al*. 2019).

In heterogeneous photocatalysis the most common catalysts are transition metal oxides and semiconductors such as of the chalcogenide type oxides (*e.g.* TiO_2, ZnO, ZrO_2, CeO_2), or sulphides (*e.g.* CdS, ZnS)), or they can be constructed by noble metal deposition coupling two semiconductors or ion-doping (Ollis and Al-Ekabi, 1993; Schiavello, 1988; Serpone and Pelizzetti, 1989; Cammarata, 2006; Okpala, 2013). In order to obtain an improved photo-absorption efficiency for pure semiconductors, various morphological micro/nanostructures with large specific surface areas, such as hollow microsphere, nanoflower, nanosheet and nanotube, have been synthesized (Belver *et al*. 2019).

Semiconductors act as sensitizers for light induced redox processes due to their electronic structure. As shown in the **Figure 1.2**, a semiconductor is characterized by a valence band (VB) fully filled with electrons and an empty conduction band (CB) at low temperature (Hoffmann *et al*. 1995; Belver *et al*. 2019).

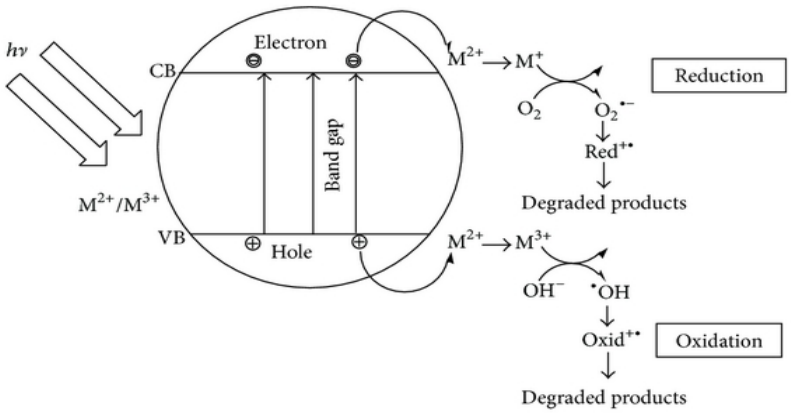


Figure 1.2 Simplification of the photocatalytic process evolving a semiconductor (Hoffmann *et al*. 1995)

The energy region between VB and CB has no electron states and is called as the bandgap. The semiconductor is illuminated by photons absorbing them with energy equal to or greater than its band gap, thus each photon of requisite energy (*i.e.* wavelength) that hits an electron in the occupied VB of the semiconductor atom can excite that electron to the unoccupied CB. Meanwhile, leaving positive VB holes; thus, photogenerated electron-hole pairs are produced **Figure 1.2** (Ahmed and Ollis, 1984; Schiavello and Sclafani, 1989; Hoffmann *et al*. 1995; Belver *et al*. 2019). The formed photoelectrons from the CB (e^-) possess strong reduction capacity acting as reducing agents and the positive photoholes (h^+) from the VB have strong oxidation capacity (oxidizing agents) which diffuse on the surfaces of the semiconductor particles and react with water, oxygen and organic pollutants in water (Ahmed and Ollis, 1984; Hoffmann *et al*. 1995; Bagheri and Julkapli, 2015; Belver *et al*. 2019). As a result of these initial reactions and the following oxidation and hydroxylation reactions by OH^- radicals, the degradation of organic pollutants via fragmentation products and finally to CO_2 and water can be expected (Hoffmann *et al*. 1995).

Despite TiO_2 be considered one of the most promising catalysts, some problems can occur when it is used in photocatalysis such as:

i) photogenerated electron–hole pairs can recombine quickly, which affects the photocatalytic efficiency, and

ii) it can be only excited by ultraviolet (UV) light, which is < 5% of solar light, because of its wide bandgap (Wu *et al*. 1999). These disadvantages of TiO_2 may result in a low photocatalytic activity in real applications. Therefore, several recent studies have focused on the preparation and modification of TiO_2 to narrow its bandgap and enhance the photocatalytic activity under visible-light radiation (Pascariu *et al*. 2019).

In the present study this approach was applied, with the synthesis of nanocomposites of lead sulphide coupled with TiO_2 that can be used as catalysts.

The nanocomposites can present advantages over the conventional composites. Cammarata (2006) observed that as with conventional particles, the properties of nanocomposites can display synergistic improvements over those of the component phases individually.

Okpala (2013) explained that the synthesis of nanocomposites constitutes a “rapidly expanding field which is generating many exciting new materials with novel properties.” Nanocomposites can be derived by combining properties from the parent constituents into a single material, from this combination there is also the possibility of

rising new properties which are unknown in the parent constituent materials (Okpala, 2013). In mechanical terms, nanocomposites differ from conventional composite materials due to the exceptionally high surface to volume ratio of the reinforcing phase and/or its exceptionally high aspect ratio, *i.e.* particles with high length/diameter ratio, such as nanotubes, nanorods, and platelets depending on their aspect ratio. The reinforcing materials can be made up of particles (minerals), sheets (*e.g.* exfoliated clay stacks) or fibres (*e.g.* carbon nanotubes or electrospun fibres). The area of the interface between the matrix and reinforcement phase(s) is typically in order of magnitude greater than for conventional composite materials. The matrix material properties are significantly affected in the vicinity of the reinforcement. Other kinds of nanoparticulates may result in enhanced optical properties, dielectric properties, heat resistance or mechanical properties such as stiffness, strength and resistance to wear and damage. In general, the nano reinforcement is dispersed into the matrix during processing (Okpala, 2014).

1.2.2. Solar photocatalysis

The solar-photocatalysis is an alternative and energy efficient technology for degradation of organic pollutants in water. Advantages of photocatalysis system for wastewater treatment applications include:

- uses the ambient oxygen as natural and simple oxidant agent, while other advanced oxidation technologies use expensive oxidizing chemicals, especially those using oxidants such as hydrogen peroxide and ozone;
- reaction 106 to 1012 times faster than alternative oxidizing agents;
- needs low energy technology without the use of lamps (sun light) or with the use of low energy lamps by artificial visible light emission;
- can be combined with other technologies (nanofiltration or biological treatment);
- can be applied for both slurry and immobilized reactor;
- can be used in many fields of application in water treatment (drinking water, ground water, process water);
- can degrade a wide range of organic pollutants like pharmaceuticals or personal care products in water at very low concentrations;
-useful for pre-treatment of water containing non-biodegradable contaminants
- can be self-regenerated

- one of the major advantages of photocatalytic process over existing technologies is that there is no further requirement for secondary disposal methods (Bagheri and Julkapli, 2015; Ahmed and Ollis, 1984).

Furthermore, the operation is normally performed at or near room temperature and pressure, which is a considerable environmentally friendly process (Sahar *et al*. 2015).

However, the main disadvantage of photocatalytic nanoparticles is their low efficiency when using the sunlight. Other disadvantages are photochemical side reactions, fast back electron transfer processes and recombination reactions, that may considerably limit the efficiency of photocatalytic reactions, and also the fact that photochemical decomposition of the catalyst may further lead to the fast termination of photocatalytic cycles (Hennig and Billing, 1993).

1.3. Biodegradation

"Biodegradation is a key process in the natural attenuation (reduction or disposal) of organic/inorganic chemical compounds at hazardous waste sites" (James and Speight, 2017).

Biodegradation is the term given for the natural process(es) to the breakdown (degradation into nutrients) of organic/inorganic compounds such as pharmaceutical drugs by the biological action of a living organism (Knapp and Bromley-Challoner, 2003; James and Speight, 2017). In the environmental context, generally microorganisms namely bacteria are the most important agents of biodegradation. Although extensive degradation of some xenobiotic can occur in mammals (usually in the liver), this process is not of special importance in the degradation of environmental pollutants. The heterotrophic bacteria are often considered to be of main importance; though, the role of *fungi* is nowadays being increasingly recognized, while *algae* and *cyanobacteria* can catalyse some biodegradative processes, but their contribution seems to be only of limited importance (Knapp and Bromley-Challoner, 2003).

Bioremediation is used when physical and chemical methods of remediation may not completely remove the contaminants, leaving residual concentrations that are above the regulatory limits for water quality (James and Speight, 2017).

The most part of biodegradation systems operate under aerobic conditions (in the presence of oxygen), nevertheless systems under anaerobic conditions (in the absence of oxygen) may allow microorganisms to degrade inorganic/organic chemical compounds

that are otherwise nonresponsive to aerobic treatment and vice versa (James and Speight, 2017).

The ability of a chemical to be biodegraded is a crucial factor for understanding the risk posed by this compound on the environment. The contaminants of concern must be liable to degradation under appropriate conditions, but the success of the process depends on the ability to determine these conditions and establish them in the contaminated environment. The success of the process depends on some required site factors such as:

(i) presence of viable microbial populations which are metabolically capable;

(ii) proper environmental growth conditions, such as the presence of oxygen or its absence for aerobic and anaerobic populations, respectively;

(iii) suitable temperature conditions. This is an important variable since keeping a substance frozen or below the optimal operating temperature for bacteria can avoid biodegradation; biodegradation generally occurs at temperatures between 10°C and 35°C;

(iv) the presence of water;

(v) appropriate concentrations of nutrients and contaminants;

(vi) favourable acidity or alkalinity.

Regarding the last parameter, soil pH is extremely important because most bacteria have an optimal pH range to survive and operate; for example, the biodegradation of chemicals might be optimal at a pH 7 (neutral), but the acceptable (or optimal) pH range might be in the order of 6–8. Furthermore, this parameter can affect the availability of nutrients in soil or water. Thus, through biodegradation processes, microorganisms particularly bacteria, yeasts, molds and filamentous fungi can transform and/or metabolize various classes of toxic compounds (James and Speight, 2017).

Biodegradation can be used as a cost-effective secondary treatment in WWTPs to decrease the concentration of contaminants to acceptable concentrations. In other cases, biodegradation can be the primary treatment method followed by physical or chemical methods for final site endpoint. Also, it is the preferred process for very large areas (James and Speight, 2017).

1.3.1. Wastewater Treatment Plant – Biological Process

Conventional WWTPs employing activated sludge treatment are designed to remove bulk constituents, like organic matter, nitrogen and phosphorous, which are present at

concentrations of mg/L. This process is commonly used to treat domestic and industrial wastewater aiming the removal of organic compounds from sewage influent **(Figure 1.3**) (Koh *et al*. 2008; Wilt, 2018).

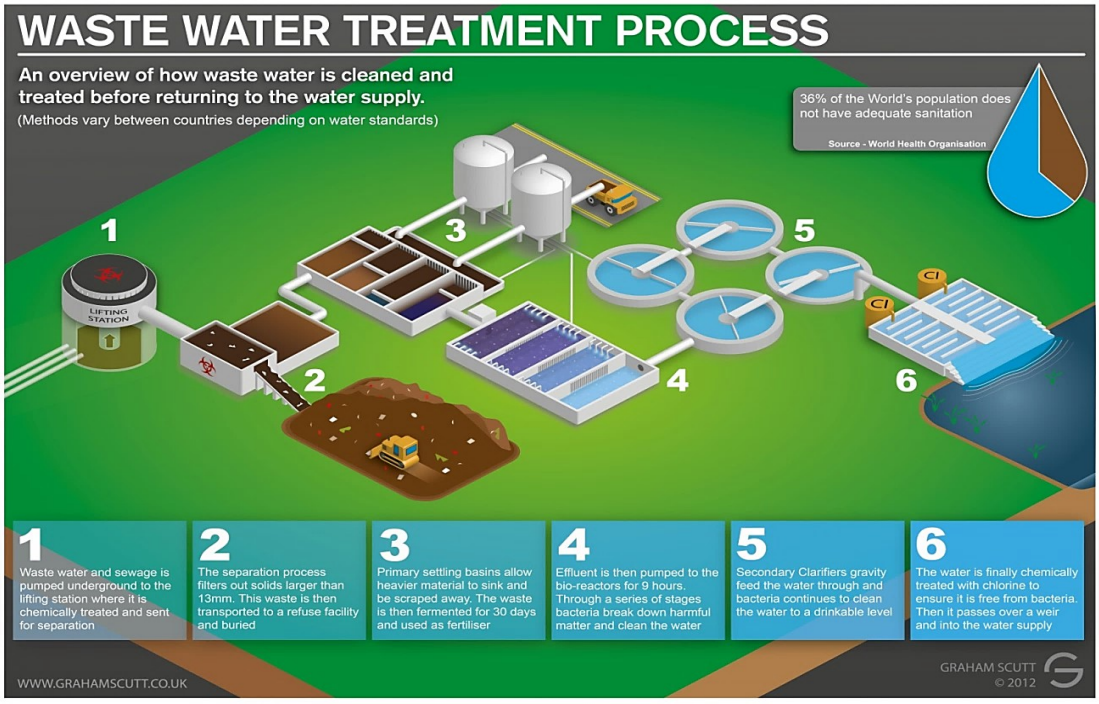


Figure 1.3 A representative infographic describing the process of how wastewater and sewage is treated for its emission into the receiving water source (adapted from http://www.grahamscutt.co.uk/)

In WWTP processes, biodegradation can be partial or complete. When biodegradation is complete the substances are converted to inorganic substances in a process of mineralization. In the case of complete breakdown, the final products are water and carbon dioxide, whereas partial breakdown results in the transformation of pharmaceuticals into metabolites (Palma *et al*. 2018).

Although WWTPs are not designed to remove pharmaceutical drugs, found at low or residual concentrations from nanograms to micrograms per litre, several researchers reported their removal suggesting the high potential of biological treatment (Joss *et al*. 2006; Monteiro and Boxall, 2010; Shon *et al*. 2006; Wilt, 2018).

Also, WWTPs are the main responsible for the effluent discharges to the receiving waters; thus, avoiding pharmaceutical emissions into the environment regarding the quality and safety of water in which respects the Directive 98/15/EC (Chelliapan and Sallis, 2011; Hamza *et al*. 2016).

The differences in drug removal efficiencies that are found among different WWTPs are generally linked to the WWTP design (Cirja *et al*. 2008). Thus, researchers suggest that

pharmaceutical treatment can be improved when its removal would be regarded as a design parameter. Several WWTP characteristics and design parameters have therefore been studied for a better understanding of the underlying pharmaceutical removal mechanisms (Wilt, 2018).

Pharmaceutical removal from WWTPs is largely dependent upon chemical structure and physicochemical properties of the drug itself, the treatment method, operational conditions such as redox conditions, hydraulic retention time, sludge retention time, the use of biofilm carrier and it also depends on the season (Verlicchi *et al*. 2012; Gracia-Lor *et al*. 2012; Jelic *et al*. 2011; Yang *et al*. 2017; Wilt, 2018).

Biological treatment is greatly favoured for its high efficiency, low cost and utilization of the diversity of the naturally occurring microbial communities metabolically able to transform or remove compounds to nontoxic products (Hu *et al*. 2012).

Several research studies have demonstrated the success of various methods in the removal of some pharmaceutical drugs such as aerobic and anaerobic (redox conditions) biodegradation and sorption to WWTP sludge (Ternes *et al*. 2004; Kujawa-Roeleveld, 2008; Hamza *et al*. 2016; Ahmed *et al*. 2017).

Some authors referred that within a certain WWTP, pharmaceutical drugs are commonly slightly removed (<5%) while others show removal percentages of over 95% (Camacho-Muñoz, 2012; Verlicchi *et al*. 2012; Wilt, 2018).

The typical design of WWTP do not comprise the removal of the thousands of highly complex molecules from the broad spectrum of pharmaceuticals presents in wastewater. In fact, numerous pharmaceuticals and/or their human metabolites and transformation products are discharged into the environment (Wilt, 2018) therefore being detected in water resources such as rivers. This suggests that (i) often the biodegradable pharmaceuticals are rather transformed than mineralized, leading to a poor removal and/or (ii) the degree of human use and pharmaceuticals disposal in sewage overwhelm their effective removal by conventional sewage treatment (Peake *et al*. 2015; Wilt, 2018).

There is a lack of information about the environmental behaviour of the human metabolites and transformation products formed from the physicochemical and microbial removal of pharmaceuticals in sewage treatment facilities (Peake *et al*. 2015). Yet, given that these metabolites can be persistent and more toxic than the parent compound, it is essential to determine their fate in biological systems (Mbokou *et al*. 2016; Pereira *et al*. 2016; Santos *et al*. 2013; Peake *et al*. 2015) and, hence, the need to

search for new and effective ways to degrade or remove them from wastewater treatments is of high importance. In this regard, biodegradation may represent an economical effective solution (Wu *et al*. 2012), though (photo)chemical degradation has been also extensively studied (Fatta-Kassinos *et al*. 2011).

Photocatalysis and biodegradation are known complementary processes for the removal of various organic contaminants such as quinolone, pyridine and pesticides from water (Wang *et al*. 2015; Yan *et al*. 2013; Zhang *et al*. 2012; Zhang *et al*. 2014). For a limited number of individual pharmaceuticals this combination has been studied (Gómez-Pacheco *et al*. 2011; Pan *et al*. 2014; Xiong *et al*. 2017), but for pharmaceutical mixtures the effectiveness of the combined treatment remains unknown. Moreover, photocatalytic processes commonly require considerable amounts of energy inputs and are thereby not very sustainable. Hence, it is of great importance to improve the resource efficiency, *e.g.* in an energy efficient combination with biological treatment, before this treatment process can find its way to large scale application in wastewater treatment (Wilt, 2018)

1.4. Pharmaceutical drugs under study

The present work is focused on the:

(i) photodegradation of chloramphenicol and paracetamol;

(ii) search of bacterial biodegrading communities and bacterial isolates with ability to degrade paracetamol, fluoxetine, 17α-ethynilestradiol;

(iii) implementation of a granular system in which ibuprofen degradation was tested during its optimization.

1.4.1. Chloramphenicol

Antibiotics are considered EPs belonging to a group of antimicrobial compounds commonly used to treat diseases caused by microorganisms (Martinez, 2009).

The presence of antibiotics in the environment has attracted attention within the scientific community due to their high consumption, low biodegradability, toxic effects, and contribution to the development of resistant bacteria (Yu *et al*. 2009; Trovó *et al*. 2014; Deng *et al*. 2016; Podder *et al*. 2018).

Chloramphenicol (CAP) (**Table 1.1**) (presented in the **page 23**) is a broad-spectrum antibiotic, a D-threo isomer of a small molecule, consisting of a p-nitrobenzene ring

connected to a dichloroacetyl tail through a 2-amino-1,3-propanediol moiety (Dinos *et al*. 2016).

The natural source of its isolation is *Streptomyces venezualae* in 1947 *(*Das and Patra, 2017) but is now produced synthetically (Wongtavatchai *et al*. 2005). CAP acts principally by binding to the 50S subunit of the bacterial ribosomes restraining the protein chain elongation by peptidyl transferase inhibition (Van Bambeke *et al*. 2017; Das and Patra, 2017). However, it can also interact with mitochondrial ribosomes of eukaryotic cells, which results in its toxicity (Van Bambeke *et al*. 2017).

Chloramphenicol is highly lipid soluble and has good penetration into many body tissues, penetrates well into pleural and ascitic fluids and also crosses the placenta and can be found in breast milk (Moffa and Brook, 2015).

Chloramphenicol was first used for the treatment of typhoid but with the global presence of multiple drug-resistant *Salmonella Typhi* it loses its clinical value. It is widely used for the treatment of bacterial conjunctivitis, staphylococcal brain abscesses (because of excellent blood–brain barrier penetration), and meningitis. It is used to treat infections caused by tetracycline-resistant cholera and vancomycin resistant *enterococcus* (*VER*) (Das and Patra, 2017). CAP has a historical in veterinary uses in all major food-producing animals and with current uses in humans and companion animals (Wongtavatchai *et al*. 2005).

Acquired resistance occurs in many species, especially where CAP is in common use such as *Pseudomonas aeruginosa*, *Escherichia*, *Klebsiella*, *Enterobacter*, *Salmonella* and *Proteus* now show patterns of plasmid-mediated resistance. *Mycobacterium* and *Nocardia* are resistant. All anaerobic bacteria are inhibited by chloramphenicol at usual therapeutic concentrations (Jill *et al*. 2008).

CAP is an antibiotic drug, commonly used as a representative antibiotic, due to its effectivity against both gram-positive and gram-negative bacteria (Giri and Golder, 2018). CAP is, in certain susceptible individuals, associated with serious toxic effects in humans including bone marrow depression, particularly severe in the form of fatal aplastic anemia, produces a reversible suppression of erythropoiesis after several days of therapy, can cause severe agranulocytosis and grey baby syndrome (Woodward, 2004; Grayson *et al*. 2017). Also, concerns have been expressed about the genotoxicity of CAP and its metabolites, its embryo- and fetotoxicity, its carcinogenic potential in humans (Wongtavatchai *et al*. 2005).

CAP was found in wastewater influents up to 3 ng/mL and in sea water samples near aquaculture sites at concentrations up to 15.6 ng/mL (Tahrani *et al*. 2016). In Asian countries including China, CAP was detected in concentrations between 26 to 2430 ng/L in influents and from 3 to 1050 ng/L in effluents of sewage treatment plants (Giri and Golder, 2018). CAP concentrations found in municipal sewage and in the Nanming River of Guiyang City, China were up to 47.4 μg/L and 19.0 μg/L, respectively (Liu *et al*. 2009).

Nowadays it is well known that antibiotics contamination and accumulation can cause hazardous effects in aquatic organisms such as arthropathy, nephropathy, damage to the central nervous system and spermatogenesis, mutagenic and light sensitivity and presents a significant risk to environmental and human health, even at low concentrations (Kümmerer *et al*. 2000; Gao *et al*. 2012; Bouki *et al*. 2013; Tahrani *et al*. 2016).

Particularly for CAP, an antibiotic, the best option was to perform photocatalysis instead of biodegradation, since it is known to be poorly biodegraded and also it was not intended to find or evolve antibiotic resistant microorganisms, due to the negative impact that this may have to the environment and public health.

1.4.2. Paracetamol

Paracetamol (N-(4-hydroxyphenyl)acetamide), or acetaminophen, (**Table 1.1**) (presented in the page 23) is a member of the class of phenols and acetamides. It is derived from 4-aminophenol in which one of the hydrogens attached to the amino group has been replaced by an acetyl group. Paracetamol is a widely used nonprescription non-narcotic analgesic and an antipyretic for mild-to-moderate pain and fever (Jóźwiak-Bebenista and Nowak, 2014; NCBI, 2019).

It is the drug of choice in patients that cannot be treated with a non-steroidal anti-inflammatory drug (NSAID), such as people with bronchial asthma, peptic ulcer disease, haemophilia, salicylate-sensitized people, children under 12 years of age, pregnant or breastfeeding women (Jóźwiak-Bebenista and Nowak, 2014). Harmless at low doses, it has direct hepatotoxic potential when taken as an overdose and can cause acute liver injury and death from acute liver failure (NCBI, 2019).

Paracetamol is considered an EPs due to its widespread global use and the fact it is readily accumulated in aquatic ecosystems with adverse effects (De Gusseme *et al*. 2011; Mathai *et al*. 2012; Mbokou *et al*. 2016).

Concentrations up to 180 µg/L have been reported in WWTP influents while in the effluents the concentrations reported were up to 0.305 µg/L in systems with aerobic tanks and up to 13 µg/L in wetlands (*e.g*. Roberts and Thomas, 2006; Papageorgiou *et al*. 2016; Vymazal *et al*. 2017). In Portugal, the highest value recorded in wastewater effluent was 32 µg/L (Pereira *et al*. 2016). Paracetamol's main metabolite paracetamol-glucuronide enters the environment via human excretion and has been detected in WWTP effluent at levels of up to 462 µg/L (Santos *et al*. 2013; Peake *et al*. 2015). Paracetamol glucuronide (phase I), and its principal transformation product, 4-aminophenol (phase II), have been detected in Portuguese river and surface waters at concentrations of 3.57 µg/L and 1.63 µg/L (Santos *et al*. 2013).

Some authors demonstrated that paracetamol was efficiently removed by bacteria namely by a nitrifying bacterial culture and by two isolated bacteria *Delftia tsuruhatensis* and *Pseudomonas aeruginosa* (De Gusseme *et al*. 2011; Hu *et al*. 2012; Karaman *et al*. 2016). Also, Zhang *et al*. (2013) have isolated from these microbial aggregates three bacterial strains of the genera *Stenotrophomonas* and *Pseudomonas* capable of using paracetamol as their sole carbon, nitrogen and energy sources and proposed metabolic pathways for the degradation of this drug (**Figure 1.4**).

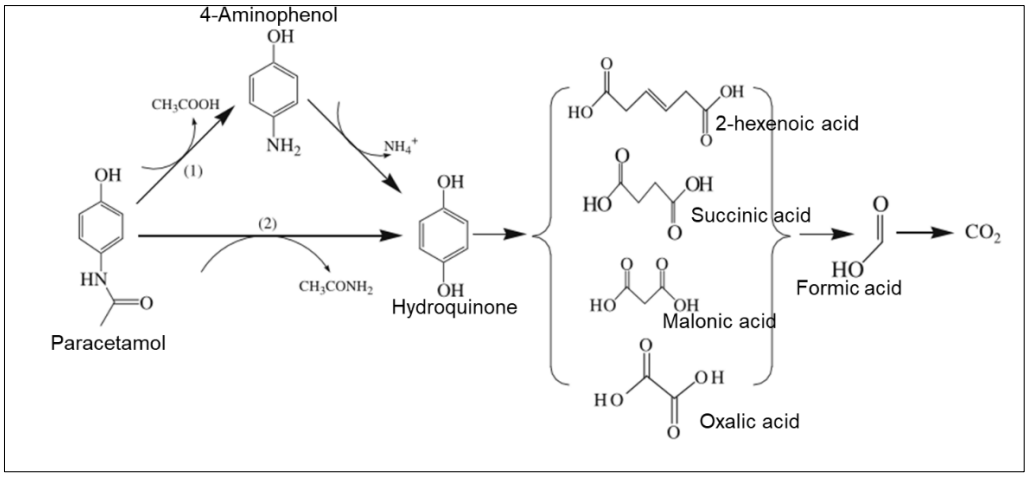


Figure 1.4 Proposed pathway for the degradation of paracetamol (adapted from Zhang *et al*. 2013). The microbial degradation of paracetamol can produce 4-aminophenol, hydroquinone, acetic acid, formic acid, benzoquinone, or oxalic acid

Zhang *et al*. 2013 identified 4-aminophenol and hydroquinone as the two main metabolites of the biodegradation pathway of paracetamol. They suggest that the paracetamol biodegradation pathway is constituted by the following steps:

(1) paracetamol is initially catalysed by an amidohydrolase with the release of acetate to yield 4-aminophenol, in which the amino group is then replaced by a hydroxyl group to form hydroquinone, subsequent to ring fission.
(2) In the second instance, the initial hydroxylation of paracetamol could potentially be catalysed by a hydrolytic enzyme to produce hydroquinone with a release of acetamide, which could be further converted into oxalic acid. Following the two aromatic intermediate compounds, five carboxylic acids were successfully isolated and identified, suggesting that the two aromatic compounds could be precursors of these carboxylic acids. Nitrite and nitrate could be detected, indicating –CO–NH_2 group of paracetamol could be easily mineralized to nitrite and nitrate by the isolated strains. The observed nitrite and nitrate could also be taken as an indicator for the biodegradation and mineralization of paracetamol (Zhang *et al*. 2013).
Also, photodegradation of paracetamol was performed. Jallouli *et al*. (2017) studied the photodegradation of paracetamol using TiO_2 P25 as a photocatalyst in slurry and under UV light. In the presence of TiO_2 P25 paracetamol was degraded in 150 min. Therefore, a very fast photodegradation of this drug and effective mineralization occurred. In this process more than 90% of the drug was degraded under UV irradiation. The main intermediate products generated during the photocatalytic reaction were found to be hydroquinone, benzoquinone, p-nitrophenol, and 1,2,4-trihydroxybenzene (Jallouli *et al*. 2017).

1.4.3. Fluoxetine

Fluoxetine (FLX) (N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine) hydrochloride (**Table 1.1**) (presented in the **page 23)** is the first agent of the class of antidepressants known as selective serotonin-reuptake inhibitors (SSRIs). Fluoxetine is an aromatic ether, a fluorinated pharmaceutical consisting of 4-trifluoromethylphenol (in which the hydrogen of the phenolic hydroxy group is replaced by a 3-(methylamino)-1-phenylpropyl group. It is a member of (trifluoromethyl)benzenes and a secondary amino compound (NCBI, 2019). Fluoxetine is constituted by a racemic mixture (50/50) of (R)-FLX and (S)-FLX enantiomers with equivalent pharmacologic activity, prescribed for the treatment of depression and is one of the most dispensed drugs in the world (Stevens and Wrighton, 1993).
Despite distinct structural differences between compounds of this class, SSRIs possess similar pharmacological activity. As with other antidepressant agents, several weeks of

therapy may be required before a clinical effect is seen. SSRIs are potent inhibitors of neuronal serotonin reuptake. Fluoxetine may be used to treat major depressive disorder, moderate to severe bulimia nervosa, obsessive-compulsive disorder, premenstrual dysphoric disorder panic disorder with or without agoraphobia and in combination with olanzapine for treatment resistant or bipolar I depression. Fluoxetine is the most anorexic and stimulating SSRI (Stevens and Wrighton, 1993; NCBI, 2019).

In humans FLX is extensively metabolized in the liver. The only identified active metabolite, norfluoxetine (NFLX), is formed by demethylation of FLX (**Figure 1.5**) (Whirl-Carrillo *et al*. 2012). The main path of FLX excretion is via urine with less than 10% of the drug reported to be excreted in the urine as unchanged or as FLX glucuronide (Benfield *et al*. 1986). Environmental contamination with this drug and its active metabolite NFLX has been detected in sewage effluents and in surface waters (Metcalfe *et al*. 2010; Petrie *et al*. 2014).

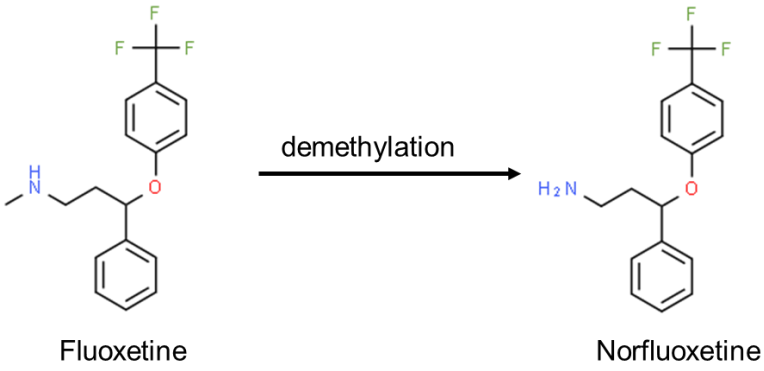


Figure 1.5 FLX metabolization into NFX by demethylation

The total concentrations of the respective enantiomers of FLX and NFLX were between 3.5 ng/L to 16 ng/L in raw wastewater and between 1.2 ng/L to 15 ng/L in treated wastewater, the same concentration ranges as found in other studies performed in Europe and in North America. Interestingly, the Predicted Environmental Concentrations (PEC) of (R,S)-FLX in raw wastewater in Spain have been determined to be in the range from 80 to 200 ng/L (Barclay *et al*. 2012).

Studies performed by Brooks *et al*. (2003) indicated that FLX adversely affects aquatic organisms at levels at least an order of magnitude higher than that reported in municipal effluent concentrations ranging from 0.3 to 1.6 nM. They found that unlike pesticides, the measured environmental pharmaceutical concentrations are not acutely toxic to aquatic organisms (Brooks *et al*. 2003). Fluoxetine exposition even at trace

concentrations can result in chronic responses of non-target biota, many of these effects remain to identify (Fong *et al*. 1998). Thus, aquatic systems in regions where municipal effluents do not benefit from upstream dilution may present maximal risk to aquatic organisms (Fong *et al*. 1998; Brooks *et al*. 2003).

Kwon and Armbrust (2006) performed ready-biodegradability experiments and they found that FLX would not be expected to be quickly biodegraded in WWTP. Their results indicated that FLX is relatively recalcitrant to hydrolysis, photolysis and microbial degradation, but that it is rapidly removed from surface waters by adsorption to sediment, where it appears to be persistent (Kwon and Armbrust, 2006). They also demonstrated that FLX was quickly dissipated from the aqueous phase in water/sediment systems, mainly by its distribution (adsorption) to sediments, therefore, decreasing the negative impact of FLX in aquatic environments (Kwon and Armbrust, 2006). NFLX was identified as the only photoproduct in a sample of synthetic humic water, which was formed by demethylation (Kwon and Armbrust, 2006).

Several other authors reported that the main mechanism for FLX removal was adsorption to biosolids/sludge (Kinney *et al*. 2006; Cartwright *et al*. 2010; Moreira *et al*. 2015). Thus, the current literature suggests that FLX and NFLX may persist in environmental sediments and both these compounds are poorly degraded by hydrolysis, biodegradation, and photolytic processes. There is still uncertainty around the environmental risks posed by FLX and NFLX (Peake *et al*. 2015).

1.4.4. 17α-Ethinylestradiol

17α-Ethinylestradiol (EE2) (**Table 1.1**) (presented in the **page 23**) is a semisynthetic hormone derived of the natural estrogen E2 (17β-estradiol and an estradiol), which is mainly used as a component of oral contraceptives (Aris *et al*. 2014). 17α-Ethynylestradiol is a 3-hydroxy steroid that is an estradiol substituted by an ethynyl group at position 17 (Saaristo *et al*. 2010).

EE2 belongs to endocrine-disrupting chemicals (EDCs) that constitute a group of organic pollutants with increasing importance due to their impact in the environment and human health (Diamanti-Kandarakis *et al*. 2009). These chemicals may interfere with the function of the endocrine system in wildlife and humans by blocking or mimicking the normal effect of hormones, affecting their synthesis or metabolism and altering hormone receptor levels (Diamanti-Kandarakis *et al*. 2009).

Estrogens are polar and hydrophilic in nature suggestive of low adsorption. The log K_{ow} (concentration ratio at equilibrium of an organic compound partitioned between an organic liquid and water) of steroid estrogens E1, E2, estriol (E3) and estrone-3-sulfate (E1-3S) are 3.4, 3.1, 2.7 and 0.95, respectively (Koh *et al*. 2008).

EE2 is largely excreted by human and animals in urine and faeces as active free forms or inactive glucuronide and sulphate conjugates (Barreiros *et al*. 2016). Thus, due to their steady release via wastewater effluents into surface waters and their high biological activity, EE2 was identified as one of the major sources of estrogenic activity in the environment causing negative effects on ecosystems and living beings, even at ng/L levels. This compound has been associated with fish feminization (synthesis and secretion of vitellogenin), reproduction and behaviour modifications, fertility reduction, increase of breast and testicular cancer in humans and promotion of abnormal reproductive processes (Barreiros *et al*. 2016; Konemann *et al*. 2018). Moreover, these chemicals have the potential to bioaccumulate and enter the food chain. For all these reasons, the European Union has recently added E2 and EE2 to a new "watch list" of emerging aquatic pollutants included in the Water Framework Directive (Barreiros *et al*. 2016; Konemann *et al*. 2018).

However, some authors found that EE2 suffer degradation by some bacteria (Ren *et al*. 2007; Yi and Harper, 2007). Examples of degradation pathways for EE2 by bacteria are described in **Figure 1.6**.

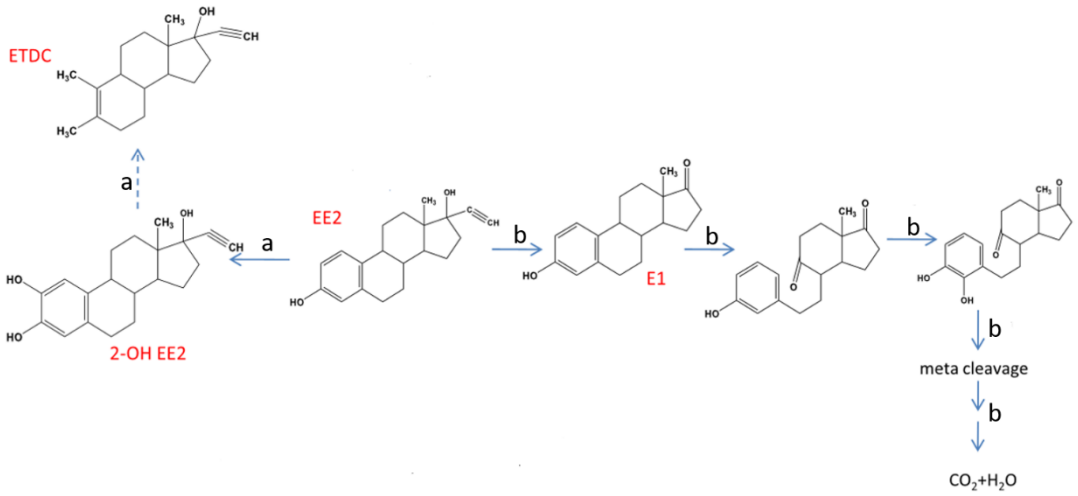


Figure 1.6 Degradation pathways of EE2 by bacteria. ETDC – (3-ethynyl-3a,6,7-trimethyl-2,3,3a,4,5,5a,8,9,9a,9b-decahydro-1*H*-cyclopenta[a]naphthalen-3-ol).
Confirmed pathways: solid lines. Uncertain pathways: dash lines (adapted from Yu *et al*. 2013)

The first steps of the different pathways are classified as:

(a) A ring C-2 hydroxylation: This was identified during a study of Yi and Harper (2007) when EE2 was degraded by an enriched nitrifying culture. In this study, 2-OH EE2 was detected, suggesting the hydroxylation of A ring at C-2 position of EE2. The ring A is the first one to suffer cleavage followed by other rings (B, C, or D rings). Their results suggested that EE2 was co-metabolically transformed by nitrifying bacteria (Yi and Harper, 2007; Yu *et al*. 2013).

b) D ring C-17 conversion to keto: Ren *et al*. (2007) identified several transformation metabolites during EE2 biodegradation by *Sphingobacterium* sp. JCR5 an EE2-utilizing isolate. The metabolites detected were 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9.17-dione, 2-hydroxy-2,4-dienevaleric acid and 2-hydroxy-2,4-diene-1,6-dioic acid. The EE2 degradation by the isolate started by oxidization of C-17 of EE2 into a ketone, E1. As shown in **Figure 1.6**, the C-9 of E1 is subsequently hydroxylated and ketonizated, resulting in cleavage of the B ring of E1. Following the hydroxylation of the A ring of the metabolite, 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9.17-dione was formed. Several subsequent degradation steps, including meta-cleavage of A ring via dioxygenase, lead to production of end products, CO_2 and water.

Despite extensive research has already been carried out investigating EE2, there is still indeterminate information regarding its biodegradability, especially the relative change in toxicity and estrogenicity and the identification of by-products.

1.4.5. Ibuprofen

Ibuprofen is (2RS)-1[4-(2-methylpropyl)phenyl]propionic acid (**Table 1.1)** (presented in the page 23), the first member of propionic acid derivatives to be introduced in 1969 as a better alternative to Aspirin (Tripathi, 2003; Bushra and Aslam, 2010). Ibuprofen is the most commonly used and frequently prescribed NSAID and it is also available as an over-the-counter medication for mild pain as analgesic and antipyretic drug (WHO, 2015; Mazaleuskaya *et al*. 2015). Its mode of action results from its non-selective inhibitory effect on cyclo-oxygenases (COX-1 and COX-2), that are involved in the synthesis of prostaglandins, reducing it, which in turn have an important role in the generation of pain, inflammation and fever (Wahbi *et al*. 2005; Bushra and Aslam, 2010).

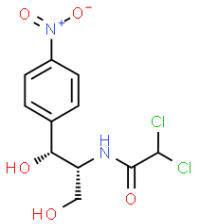
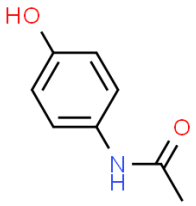
The drug is eliminated in 24 hours and is extensively metabolized in the liver with a little fraction being excreted as unchanged (Bushra and Aslam, 2010). Thus, more than 90% of an ingested dose is excreted in the urine as metabolites or their conjugates, the main metabolites are hydroxylated and carboxylated compounds (Bushra and Aslam, 2010). Therefore, around 15% of ingested IBU is excreted in urine, 25-26% as 1-hydroxyibuprofen (1-OH) and 2-hydroxyibuprofen (2-OH) and 37-46% as carboxy hydroxyibuprofen (CBX) (Vane *et al*. 1998; Weigel *et al*. 2004; Mazaleuskaya *et al*. 2015).

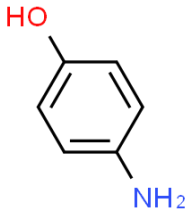
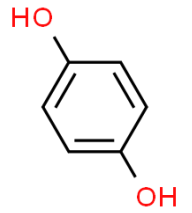
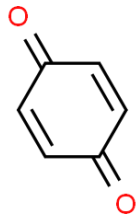
In Portugal, the IBU concentrations detected in the influents of WWTPs were 3, 3.9, 5.3, 9.80 µg/L in spring, summer, autumn and winter, respectively while the effluent concentrations were of 0.16, 0.3, 1.5, 1.8 µg/L in spring, summer, autumn and winter, respectively (Pereira *et al*. 2015; Pereira *et al*. 2016). The highest concentrations were observed in the Algarve, Alentejo and Center regions in the winter, due to population size and colder temperatures that leads to the development of colds and flu. In these conditions individual WWTPs concentrations could be as high as 30 µg/L (Pereira *et al*. 2015; Pereira *et al*. 2016). In the United Nation Strategic Approach to International Chemicals Management database, IBU is ranked as the third most detected pharmaceutical in surface, drinking and ground waters and is detected in 47 countries (aus der Beek, 2015). Several studies were performed in order to assess the environment toxicity and risk of IBU with differing results. Some have classified IBU as low risk in surface and wastewaters (Pereira *et al*. 2015; Pereira *et al*. 2016; Lolić *et al*. 2015). In contrast, several authors refer IBU as a significant environmental risk (Gamarra *et al*. 2015; Paíga *et al*. 2013; Boxall *et al*. 2014; Cleuvers, 2004; Escher *et al*. 2011). No risk assessments have been performed for the metabolites. It is supposed that they have no pharmacological activity (Besse and Garric, 2008; Lienert *et al*. 2007). Toxicity studies have proven that IBU is an aquatic environmental hazard, as a single compound or magnified with other pharmaceuticals (Cleuver, 2004; Claessens, 2013). Through its therapeutic action as a COX inhibitor, it causes toxic inhibition of growth, reproductive and inflammatory response of organisms at the trace concentrations commonly measured in the aquatic environment (Buser *et al*. 1999; Winkler *et al*. 2001; Cleuver, 2004; Ferrando-Climent *et al*. 2012; Claessens, 2013; Paíga *et al*. 2015)

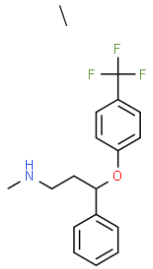
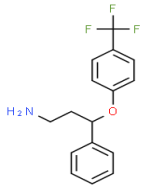
Ibuprofen removal is dependent on season and treatment employed with a high removal percentage in WWTPs ranging from 80-100% (*e.g.* Tauxe-Wuersch *et al*. 2005; Verlicchi *et al*. 2012; Pereira *et al*. 2015; Pereira *et al*. 2016). Aerobic biodegradation

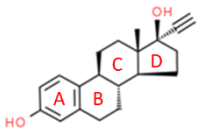
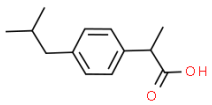
revealed to be the main removal method of these compounds in WWTPs (*e.g.* Wackett and Ellis, 1999; Kujawa-Roeleveld, 2008). Their chemical structure allows easy degradation by hydrolysis of the carboxylic acid group and carbon atoms on the aromatic ring (ring cleavage). These compounds presented low adsorption to sludge, due to their hydrophobicity and negative electrostatic charge derived from their Log K_{ow} and pKas (**Table 1.1**) (Ternes *et al*. 2004; Kujawa-Roeleveld, 2008; Martín *et al*. 2012). Despite its high removal in WWTP, IBU is considered an emerging pharmaceutical pollutant due to its widespread use, that results in its presence and of the metabolites in WWTP effluent, surface, ground and drinking waters (Kujawa-Roeleveld, 2008; Santos *et al*. 2013; Pereira *et al*. 2015; Pereira *et al*. 2016). Additionally, IBU appears to be just transformed into compounds of similar complexity and properties instead of being completely degraded. Therefore, due to the environment impact of IBU at residual concentrations it becomes mandatory to innovate on wastewater treatment processes, despite the high removal in conventional WWTPs.

Table 1.1 Characteristics of the pharmaceutical drugs under study (Ternes *et al.* 2004; Kujawa-Roeleveld, 2008; Martín *et al.* 2012; Velázquez and Nacheva, 2017; Wishart *et al*. 2018; National Center for Biotechnology Information. PubChem Database, 2019; Chemspider, 2019)

Compound	Molecular structure	ACD/ IUPAC name	Molecular formula	Molecular weight (g/mol)	Solubility in water (18 – 27 ºC)	pKa	K_{biol} (L/(gSS.day)	Log K_{ow}	Toxicity	Biological function
Chloramphenicol		2,2-Dichloro-N-[(1R,2R)-1,3-dihydroxy-1-(4-nitrophenyl)-2-propanyl]acetamide	$C_{11}H_{12}Cl_2N_2O_5$	323.13	2500 mg/L Very soluble in acetone, chloroform, ethanol.	7.49	-	1.14	Organic Compoun; Drug; Anti-Bacterial Agent; Protein Synthesis Inhibitor; Metabolite; Synthetic Compound Toxin, Toxin-Target Database T3D3954	Antibacterial drug
Paracetamol		N-(4-hydroxyphenyl)acetamide	$C_8H_9NO_2$	151.16	12750 mg/L Very slightly soluble in cold water but greater solubility in hot water	9.46	50 – 80 and 106 – 204	0.46	Organic Compound; Amide; Drug; Food Toxin; Non-Narcotic; Metabolite; Synthetic Compound Toxin, Toxin-Target Database T3D2571	Analgesic; anti-inflammatory drug; antipyretic

4-aminophenol		4-aminophenol	C_6H_7NO	109.13	121000 mg/L Soluble in acetonitrile, ethyl acetate, acetone, hot water; very soluble in dimethylsulfoxide.	10.4	-	0.04	Organic Compound; Aromatic Hydrocarbon; Amine; Food Toxin; Metabolite; Cosmetic Toxin; Household Toxin; Synthetic Compound Toxin, Toxin-Target Database T3D3600	
Hydroquinone		benzene-1,4-diol	$C_6H_6O_2$	110.11	6720 mg/L Soluble in ethyl ether. Very soluble in carbon tetrachloride	9.68	-	0.59	Organic Compound; Pollutant; Food Toxin; Metabolite; Cigarette Toxin; Household Toxin; Synthetic Compound Toxin, Toxin-Target Database T3D4577	
Benzoquinone		cyclohexa-2,5-diene-1,4-dione	$C_6H_4O_2$	108.1	11100 mg/L Poor solubility in water	-	-	0.20	Organic Compound; Ketone; Pollutant; Food Toxin; Metabolite; Household	Antibacterial drug; fungicide

									Toxin; Industrial/Work place Toxin; Synthetic Compound Toxin, Toxin-Target Database T3D4578	
Fluoxetine		*N*-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy] propan-1-amine	$C_{17}H_{18}F$	309.33	1.7 mg/L Insoluble.	9.8	0.03 – 0.244 and 0.6 – 9.0	4.05	Organic Compound; Amine; Organofluoride; Ether; Drug; Selective Serotonin Reuptake Inhibitor; Antidepressant, Second-Generation; Metabolite; Serotonin Agent; Synthetic Compound; Toxin, Toxin-Target Database T3D2802	
Norfluoxetine		3-phenyl-3-[4-(trifluoromethyl)phenoxy] propan-1-amine	$C_{16}H_{16}F$	295.3	9.15 mg/L	9.77	-	4.17		

17α-Ethinylestradiol		(8*R*,9*S*,13*S*,14*S*,17*R*)-17-ethynyl-13-methyl-7,8,9,11,12,14,15,16-octahydro-6*H*-cyclopenta[a]phenanthrene-3,17-diol	$C_{20}H_{24}O_2$	296.4	11.3 mg/L Soluble in solutions of sodium hydroxide or potassium hydroxide	10.33	7 – 9	2.8 – 4.2		Antineoplastic agent; contraceptive drug; estrogen
Ibuprofen		2-[4-(2-methylpropyl)phenyl]propanoic acid	$C_{13}H_{18}$	206.281	21 mg/L Very soluble in alcohol; Readily sol in most org solvents	5.3	21 – 35 and 9 – 22	3.97	Organic Compound; Drug; Food Toxin; Analgesic, Non-Narcotic; Anti-Inflammatory Agent; Analgesic; Anti-Inflammatory Agent, Non-Steroidal; Cyclooxygenase Inhibitor; Metabolite; Synthetic Compound Toxin, Toxin-Target Database T3D2970	Anti-inflammatory drug

1.5. Aerobic granular sludge system

An aerobic granular sludge (AGS) system, a relatively recent technology, is starting to be used as an alternative to conventional activated sludge systems for wastewater treatment. The AGS system offers an interesting alternative due to its excellent physical characteristics, as it uses a biofilm composed of microbial self-immobilized cells (Beun *et al*. 1999). In comparison to the activated sludge system, AGS has a denser and stronger microbial aggregate structure, a higher biomass concentration, a better settling capacity and the ability to withstand shock loads (Tay *et al*. 2002). These characteristics are provided by the self-aggregation of microbial communities forming granules. Several studies allowed that, nowadays, different types of granules have arisen. There is the anaerobic ammonium oxidising (anammox) granule, aerobic granule, hydrogenic granule, oxygenic photogranule and many other functionally specialised granules, that brought new possibilities in wastewater treatment biotechnology (Trego *et al.* 2019).

The representation of a granular stratified structure is shown in the **Figure 1.7**.

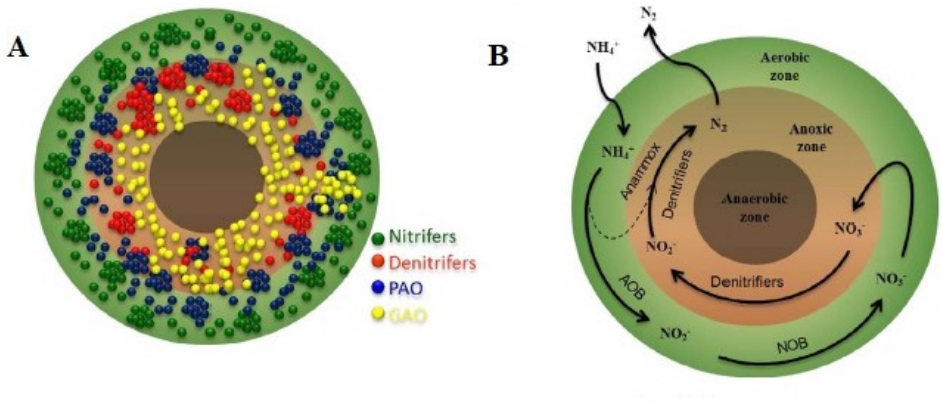


Figure 1.7 Graphical representation of granular stratified structure: (A) organization of nitrifying, denitrifying, polyphosphate accumulating organisms (PAO) and glycogen accumulating organisms (GAO); (B) nitrogen removal pathways by ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) (Adapted from Nancharaiah and Kiran Kumar Reddy, 2018)

In the granule itself there is a stratification of conversion processes, with redox zones within the granules providing aerobic and anaerobic/anoxic layers, which allows the simultaneous removal of carbon (COD), nitrogen, phosphorous (De Kreuk *et al*. 2005) and drugs such as ofloxacin, norfloxacin and ciprofloxacin (Amorim *et al*. 2014). In the outer layer, during the aeration (presence of oxygen) the growth and metabolic processes of polyphosphate accumulating organisms (PAO) which remove phosphorus and glycogen accumulating organisms (GAO) are favoured. Simultaneously, there are the ocurrence of (i) nitrification with the oxidation of nitrite to nitrate by nitrite oxidizing bacteria in the aerobic zone and (ii) denitrification with the reduction of nitrate to nitrite and subsequently to nitrogen by denitrifiers in the annoxic zone occur (**Figure 1.7**) (Nancharaiah and Kiran Kumar Reddy, 2018).

The stability and efficiency is directly dependent on the size of the granule (optimum diameter of 2-4 mm), to which dissolved oxygen saturation levels are critical. Early studies of aerobic granular systems employed mainly aerobic cycles (Beun *et al*. 2002). However, with the performed optimizations for granule stability, efficiency and effluent production respecting to EU standards, it was determined that the anaerobic/aerobic cycle is best suited. Operational differences lie in the longer length of influent feeding and shorter aeration period in the anaerobic/aerobic cycle *versus* the aerobic cycle (Adav *et al*. 2008; Beun *et al*. 2002; Ni *et al*. 2009; Lee *et al*. 2010; Lochmatter and Holliger, 2014; Awang and Shaaban, 2016).

Figure 1.8 illustrates an example of system operation in *Sequencing Batch Reactors* (SBRs) with a four-phase operational cycle.

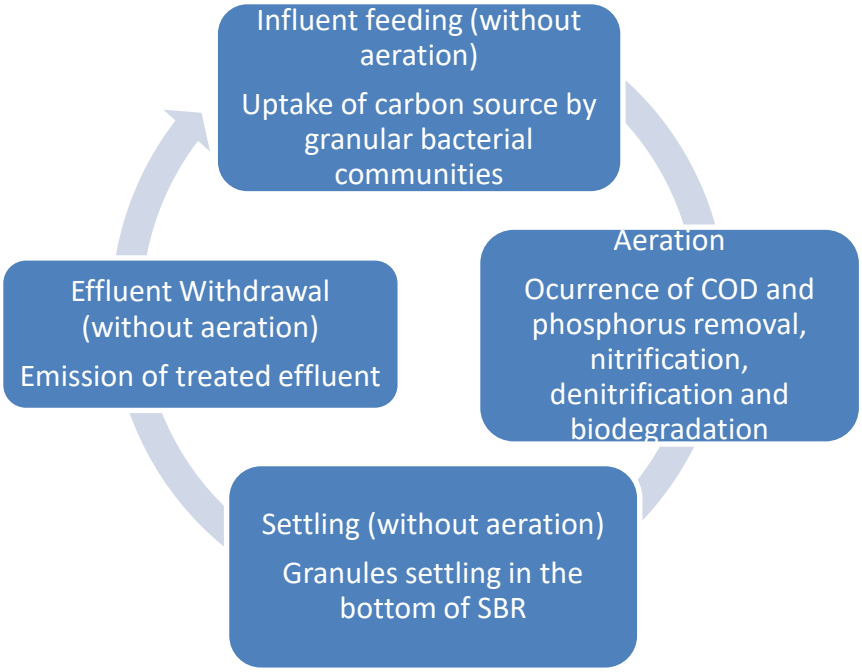


Figure 1.8 Operation cycle of aerobic SBRs (adapted from Adav *et al*. 2008)

SBRs can be started using activated sludge or already formed granules as inoculum. Several parameters can be monitored and/or measured to assess SBR efficiency and granules applicable with the desired research objective (Beun *et al*. 2002; Ni *et al*. 2009; Lee *et al*. 2010; Lochmatter and Holliger, 2014; Awang and Shaaban, 2016). Some key factors for aerobic granule formation and SBR operation are represented in **Table 1.2**.

Table 1.2 Critical factors for aerobic granule formation and SBR operation (adapted from Ennis, 2017)

Temperature	Room temperature (20-25^{0}C)	Promotes granule stability as an optimum condition for COD, nitrogen and phosphorus removal processes
Aeration	Controlled air flow that produces small air bubbles introduced at bottom of SBR	Promotes granule mixing with influent and aeration for COD, nitrogen and phosphorus removal processes
Dissolved oxygen saturation	≥ 20%	20-50% DO ensure granule stability and efficiency, promoting a larger anaerobic layer with decreased penetration depth of oxygen and promote nitrification/denitrification processes.
pH	Monitored and controlled as necessary 7.0±0.2	Optimum condition for COD, nitrogen and phosphorus removal processes
Hydraulic retention time	< 8 h	Promote microorganism proliferation and granule stability by decreasing suspended biomass growth by washout in effluent withdrawal
Influent	• Controlled flow introduced at bottom of SBR • High COD	Good interaction with granules Promote microorganism proliferation, granule formation and stability.
Effluent withdrawal	• At least 40% volume exchange ratio (percentage difference between reactor volume and volume refilled after withdrawal) • Effluent withdrawal at approximately SBR mid height	Volume exchange ratio > 40% selects for granule formation. Withdrawal height selects for fast settling particles

Several studies demonstrated the granule's biodegradation potential provided by easy adaptation to the use of different carbon sources, influent composition and operational cycle and their high resistance to toxic compounds. These characteristics are what make the granules with a great potential for application to pharmaceuticals detoxification (Jiang *et al*. 2002; Moy *et al*. 2002; Tay *et al*. 2002; Liu *et al*. 2005; Schwarzenbeck *et al.* 2005; Tay *et al*. 2005; Adav *et al*. 2009).

Regarding the novelty and potentiality if this new system of wastewater treatment it is of great importance to study its performance in terms of EPs removal.

1.6. Thesis objectives, organization and connection between charpters

The present thesis is focused on degradation studies of five extensively and globally used pharmaceutical compounds, CAP, paracetamol, FLX, EE2 and IBU:

(i) Photodegradation of CAP and paracetamol by heterogeneous photocatalysis using PbS/TiO_2 nanocomposites as catalysts;

(ii) Biodegradation of paracetamol and FLX by bacterial communities enriched from environmental samples;

(iii) Biodegradation of paracetamol, FLX and EE2 by bacterial isolates and (iv) Biodegradation of IBU by an aerobic granular SBR system.

In the case of chloramphenicol, an antibiotic, its photodegradation was tested by heterogeneous photocatalysis using lead sulphide nanocomposites as catalysts. Also, the degradation of paracetamol was tested by photocatalysis and although the degradation by this way was observed to be faster, biodegradation was preferred in lieu of photocatalysis. One of the great advantages of the biodegradation is that it occurs without the recourse to external compounds, such as the catalysts, using only the microorganisms already existing in the environment. The inocula used in the biodegradation experiments were obtained from sludge samples collected in an oxidation ditch (aerobic assays) from Faro Northwest WWTP and in a lagoon system (anaerobic assays) from Faro West WWTP. The aerobic granules were provided by Águas de Portugal Nereda® Frielas WWTP in Lisbon, Portugal.

Thus, to achieve the main objective of this work it was necessary to proceed to several steps, such as:

i. The production of lead sulphide nanoparticles and nanocomposites using TiO_2 as matrix by metals precipitation using biogenic sulphide produced by Sulphate-Reducing Bacteria (SRB). This step was performed during the Bionanomine Project (PTDC/AAG-TEC/2721/2012). The characterization of materials was carried out and the one displaying the best photocatalytic properties was used as catalyst in the photodegradation of CAP and Paracetamol (CHAPTER 2);
ii. Obtention of SRB communities from an anaerobic sludge of a lagoon system, through the enrichment of various samples and selection of the consortium with the best performance in terms of growth and sulphate reduction. The enriched community was sub-cultured and different growth conditions were tested, such as the Postgate B medium compounds which are more favourable for bacterial growth and that can interfere with the drugs under study.
iii. Optimization of analytical methods for monitoring the pollutants and their metabolites under study and their biodegradation.

iv. Batch assays were performed using sludge, from two different treatment processes as source of inoculum: an anaerobic lagoon and an aerobic reactor of Faro's WWTP, adding media with different nutritional characteristics: Postgate B, thioglycolate and MSM a mineral salt medium without any carbon source where the paracetamol was added as sole carbon source and raw wastewater which was used only for aerobic assays in order to mimic the real WWTP's conditions. Molecular characterization of the selected biodegrading communities with a putative role in drug's degradation and investigation of their dynamics by phylogenetic analysis of 16S rRNA gene. Also, metabolic products were detected (CHAPTER 3).

v. Bacteria with ability to growth and degrade the pharmaceuticals under study were isolated from the bacterial communities obtained from the WWTP sludge, where it was already detected the capacity to remove the drugs. The biodegrading ability of each drug by the isolated bacteria was studied; in the case of paracetamol, FLX and EE2, using bacterial isolates from an aerobic community. Molecular characterization of the selected biodegrading bacterial isolates by phylogenetic analysis of 16S rRNA gene was performed. The results are presented as following indicated:
Paracetamol bacterial isolates in CHAPTER 4; fluoxetine bacterial isolates in CHAPTER 6 and EE2 bacterial isolates in CHAPTER 7.

vi. Experiments with this community were carried out in Postgate B for SRB with and without lactate, and in MSM media, the last one a mineral salt medium without any carbon source, where the FLX was added as sole carbon source, under anaerobic conditions, in order to test the bacterial ability to biodegrade that drug. The metabolic products resulting from this biodegradation process were also analysed (CHAPTER 5).

vii. The introduction of a granular sludge system in these studies was suggested by Águas do Algarve - Águas de Portugal Group and was justified due to the fact that this system is being installed at the WWTP of Faro East. Thus, some work was done in the design of a microbial granular system and in its start-up in a lab-scale. Despite the optimization of the granular system still in progress, the degradation of IBU was tested. The optimization of a granular system is still ongoing for further studies on pharmaceuticals degradation (CHAPTER 8).

In general, this investigation is intended to contribute for a better knowledge on the populations that can be involved in the degradation of the pharmaceutical emerging pollutants under study, aiming in a certain away for its control through the improvement of the existing treatment technologies, or by the development of new, efficient and economical strategies to minimize their impact on the environment.

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CHAPTER 2

Photodegradation of chloramphenicol and paracetamol using PbS/TiO_2 nanocomposites produced by green synthesis

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ABSTRACT

The present study describes the photocatalytic potential of the successfully synthesized nanocrystalline PbS/TiO_2 nanocomposites, in the photodegradation of chloramphenicol and paracetamol. PbS and PbS/TiO_2 nanoparticles were synthesized using biological sulphide produced by Sulphate Reducing Bacteria in batch cultures, yielding a complete metal precipitation (~100%). The PbS and PbS/TiO_2 composites obtained using sulphide generated in batch cultures have an average particle size ranging from 17 to 25 nm and 15 to 20 nm, respectively. The produced particles presented crystalline cubic structure. The specific surface area of TiO_2 and PbS/TiO_2 were estimated to be 46.559 m^2/g and 38.005 m^2/g, respectively. Chloramphenicol removal by photolysis was of about 61% after 60 min of Hg irradiation and 36% under sunlight exposition. Chloramphenicol photocatalysis using PbS/TiO_2 as catalyst was successfully performed, in a photoreactor (Hg medium pressure, 450 W) and under solar exposition with a high drug removal efficiency of 96% and 93%, after 60 min and 240 min of irradiation, respectively. Drug's photodegradation using TiO_2 as catalyst was of 98%, for both Hg and Sunlight irradiation (UV index ranging 7 to 8) after 60 min and 240 min, respectively. Paracetamol removal by photolysis was about 18%. Drug's photocatalytic degradation using PbS/TiO_2 was successfully performed under sunlight exposition with a high removal efficiency of 93%, while in the presence of TiO_2 the removal of the drug was complete, after 235 min irradiation.

Keywords: Green synthesis; SRB; PbS nanoparticles; PbS/TiO_2 nanocomposites; Pollutants photodegradation

1. INTRODUCTION

The concern with the increasing environmental pollution especially in aquatic resources leads the researchers to find new strategies and technologies to overcome this problem. Nanomaterials namely nanocrystalline semiconductors, such as lead sulphide (PbS) integrate a new class of materials with numerous well-known applications specially as photocatalysts and as additives to solar panels (Gribov *et al*. 2017; Furasova *et al*. 2018). Thus, there is a necessity to implement new and sustainable methods for nanoparticles synthesis for further use.

The use of Sulphate-Reducing Bacteria (SRB) is often described in bioremediation of acid mine drainage (AMD) effluents, which contain high concentrations of metals and sulphate (Gibert *et al*. 2002; Costa *et al*. 2005; Mandal *et al.* 2006; Costa *et al*. 2008; Bijmans *et al*. 2009; Ayangbenro *et al.* 2018). SRB can reduce sulphate into sulphide, this last one in the presence of metal ions leads to their precipitation as metal sulphides. However, depending on the metal characteristics and sulphate concentrations, these processes could generate sulphide in excess, another environmental issue that needs to be considered (Costa *et al*. 2012; Costa *et al*. 2013). In this context, the excess of sulphide generated in a bioremediation system to treat AMD from S. Domingos mine, in Portugal, has been used to synthesize semiconductor metal sulphide nanoparticles and respective nanocomposites, such as ZnS and CuS (Costa *et al*. 2012; Costa *et al*. 2013).

The present work focuses on photocatalytic potential of the newly synthesized lead sulphide and respective TiO_2 composites in batch (**Figure 2.1**).

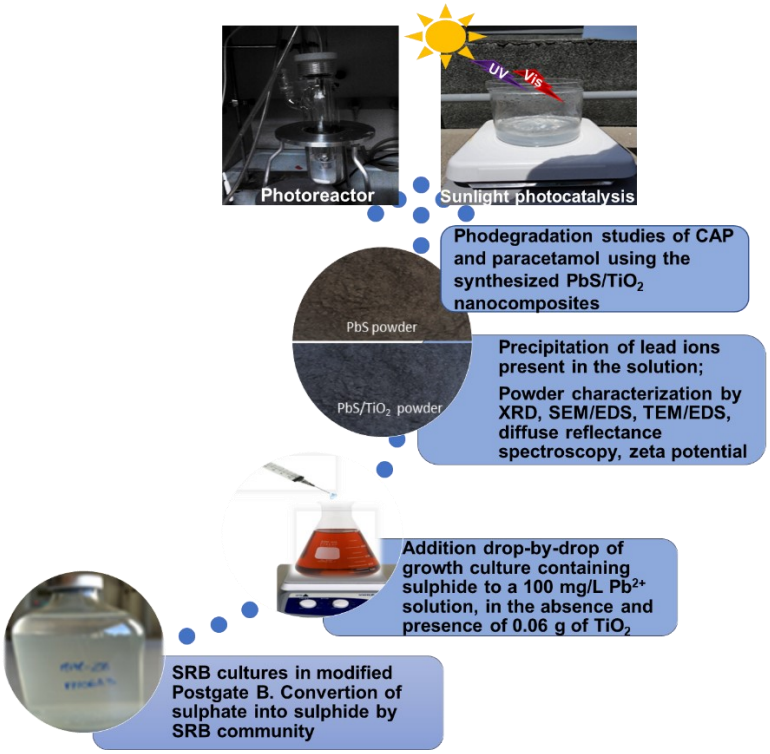


Figure 2.1 Schematic diagram of the experimental steps of PbS and PbS/TiO_2 particles synthesis in "batch" and of the performed photodegradation studies.

This process of synthesis would allow the production of metal sulphide composites exhibiting semiconductor characteristics, which can be used as heterogeneous catalysts, and could be combined with a photocatalytic process of organic pollutants detoxification using solar energy to be implemented in a wastewater treatment plant. In addition, it presents obvious advantages in terms of economy and safety, due to the production of functional materials from a toxic waste.

Moreover, the simplicity of the process here described presents an attractive alternative route to most of the chemical methods described in the literature for the synthesis of metal sulphide nanoparticles, which include chemical synthesis in aqueous medium (Bhatt *et al*. 2011) or with *in situ* chemically generated H_2S (Slonopas *et al*. 2015), vacuum deposition (Fainer *et al*. 1996), electrochemical deposition (Zhang *et al*. 2013), λ-ray irradiation (Changqi *et al*. 2003), among many others.

Also, the transition from coarse-grained to nanostructured PbS has considerably extended the scope of its application, especially in those cases where the particle size is comparable with (or less than) the lead sulphide exciton radius, which is 18 nm (Sadovnikov *et al*. 2018).

Lead sulphide (PbS) is one of the most demanded narrow-gap semiconductors which is used in a wide number of devices such as high efficiency solar cells, photodetectors operating in a broad wavelength range [from infrared (IR) to ultraviolet (UV)], thermoelectric transducers, optical switches and light emitting diodes (Fainer *et al*. 1996; Changqi *et al*. 2003; Zhang *et al*. 2013; Slonopas *et al*. 2015; Sadovnikov *et al*. 2018). PbS nanocomposites were also studied as catalysts for photodegradation of organic pollutants (Cui *et al*. 2013; Zhang *et al*. 2013; Ullah *et al*. 2014; Azimi and Nezamzadeh-Ejhieh, 2015).

As a green and sustainable technology, heterogeneous photocatalysis using semiconductors activated by visible light (320 - 750 nm) as catalysts to transform photons energy into charge carriers, has become of a great importance over the past decades due of its potential to address energy and environmental problems through the treatment/removal of a wide variety of organic contaminants using solar energy (Herrmann, 1999; Wen *et al*. 2015; Mousavi *et al.* 2017; Ameta *et al*. 2018; Pirhashemi, Habibi-Yangjeh and Pouran, 2018; Akhundi *et al.* 2019).

Photocatalysis presents several advantages over other pollutants treatment methods, like the use of inert semiconductors; the reaction can be performed at room temperature, and the organic compounds suffer oxidation even at low concentrations (Tang *et al*. 2004). For example, photocatalysts such as titanium dioxide (TiO_2) have been used with some remarkable commercial success in the production of reactive oxygen species, like hydroxyl radicals, from redox reactions of hydroxide ions and water molecules with the purpose of degrading organic/inorganic pollutants such as pharmaceutical drugs and inactivating pathogenic microorganisms under UV irradiation (Fernández *et al*. 2002; Muggli *et al.* 2002; Tang *et al.* 2004; Chatterjee and Dasgupta, 2005; Kim

and Kim, 2012; Sökmen *et al.* 2017). However, the relatively wide bandgap of TiO_2 (3.2 eV) limits further applications of the material in the visible-light region ($\lambda > 400$ nm) (Tang, Zou and Ye, 2004). Thus, to ensure the efficient utilization of visible light, the largest proportion of the solar spectrum and artificial light sources, the development of photocatalysts with high activity under a wide range of visible light irradiation is mandatory. For example, several studies were performed in order to investigate the photocatalytic degradation of diverse organic pollutants. It was observed that coupling PbS/TiO_2 (Slonopas *et al.* 2015), TiO_2/Ag_2CrO_4 (Feizpoor *et al.* 2017), TiO_2/carbon dots (CDs)/polyaniline (PANI) (Feizpoor *et al.* 2018) and TiO_2/Fe_3O_4/$CoWO_4$ (Feizpoor *et al.* 2018) extended the photocatalytic response of these semiconductors to the visible region, enhancing the activity in pollutants degradation, such as methylene blue, methyl orange, fuchsine and rhodamine dyes (Chatterjee and Dasgupta, 2005; Kim and Kim, 2012; Slonopas *et al.* 2015; Feizpoor *et al.* 2017; Sökmen *et al.* 2017; Feizpoor *et al.* 2018; Feizpoor and Habibi-Yangjeh, 2018; Sedaghati *et al.* 2019; Zarezadeh *et al.* 2019).

Antibiotics considered emerging pollutants belong to a group of antimicrobial compounds commonly used to treat diseases caused by microorganisms (Martinez, 2009). The presence of antibiotics in the environment has attracted attention within the scientific community due to their high consumption, low biodegradability, toxic effects and contribution to the development of resistant bacteria in aquatic systems (Yu *et al*. 2009; Trovó *et al*. 2014; Deng *et al*. 2016; Podder *et al*. 2018). The municipal wastewater treatment plants have been identified as a primary source responsible for the introduction of antibiotics in the aquatic environment, due to the limitations of the treatment processes and consequently a poor removal of these compounds. Thus, the antibiotics and their metabolites are continuously entering in the environment (Parsley *et al*. 2010). Their elimination in the environment can occur by biotic (biodegradation) or non-biotic (sorption, hydrolysis, photolysis, oxidation and reduction) processes. It should be highlighted that antibiotics are difficult to remove by biodegradation (Trovó *et al*. 2014). Moreover, the non-biotic elimination by sorption depends on the physicochemical properties of the target-compound. Thus, among these alternatives of elimination, the photodegradation (*e.g.* heterogeneous photocatalysis process) can be considered as the main process of elimination of these compounds in the effluents from wastewater treatment plants or other water bodies such as surface waters (Yang *et al*. 2008; Trovó *et al*. 2014; Elsaliby *et al*. 2016).

Chloramphenicol (CAP) is an antibiotic drug, commonly used as a representative antibiotic, which is effective against both gram-positive and gram-negative bacteria, and its mechanism of action is through the inhibition of the protein synthesis by the microorganism (Giri and Golder, 2018). CAP is, in certain susceptible individuals, associated with serious toxic effects in humans including bone marrow depression, particularly severe in the form of fatal aplastic anemia (Woodward, 2004).

CAP was found in wastewater influents up to 3 ng/mL and in sea water samples near aquaculture sites at concentrations up to 15.6 ng/mL (Tahrani *et al*. 2016). In Asian countries including China, CAP was detected in concentrations between 26 to 2430 ng/L in influents and from 3 to 1050 ng/L in effluents of sewage treatment plants (Giri and Golder, 2018). CAP concentrations found in municipal sewage and in the Nanming River of Guiyang City, China, were up to 47.4 μg/L and19.0 μg/L, respectively (Liu *et al*. 2009).

Several studies demonstrated that antibiotics present a critical risk to the environment and human health, even at residual concentrations. Antibiotics contamination and accumulation can cause harmful effects in aquatic organisms and humans, such as damage to the central nervous system, mutagenic effects, arthropathy, nephropathy, and light sensitivity (*e.g.* Tahrani *et al*. 2016; Kümmerer *et al*. 2000; Gao *et al*. 2012; Bouki *et al*. 2013).

Paracetamol (N-(4-hydroxyphenyl)acetamide), or acetaminophen, is the most commonly used analgesic and antipyretic drug; due to its worldwide spread, fast accumulation and hazardous effects in aquatic ecosystems, it is considered an emerging pollutant (De Gusseme *et al*. 2011; Wu *et al*. 2012; Mbokou *et al*. 2016; Żur *et al.* 2018). For example, among the surveyed systems in Czech Republic's constructed wetlands, high concentrations of paracetamol up to 180 μg/L were found in wastewater treatment plant's (WWTP) influents, while up to 13 μg/L of the drug was detected in effluents (Vymazal *et al*. 2017). In Portugal, the highest concentration of paracetamol detected in wastewater effluent was of 32 μg/L Pereira *et al*. 2016).

In several research studies, paracetamol displayed a high removal efficiency after the wastewater treatment, despite this drug was found in surface waters in concentrations ranging from 110 to 10,000 ng/L (Wilkinson *et al*. 2017) and in the western Mediterranean Sea at concentrations ranging from 0.468 to 1.70 ng/L (Brumovský *et al*. 2017; Palma *et al*. 2018). Thus, the detection of paracetamol in the hydric resources indicates that the intensity of human use and subsequent disposal in sewage exceed their effective removal by conventional wastewater treatment (Peake *et al*. 2015).

Solar heterogeneous photocatalysis (using semiconductors) is a technology that offers an energetic cost-effective alternative to remove organic pollutants, such as pharmaceuticals or personal care products, from water using the oxygen present in the air as oxidant agent; this process can be combined with other technologies such as nanofiltration or biological treatment and can also be applied in several water treatment areas (drinking water, ground water, process water) (Engeldinger *et al.* 2008).

Hence, in the present work, beside the innovative synthesis process and the characterization of the biologically synthesized PbS particles and TiO_2 composites, their potential as catalysts in the photodegradation of CAP and paracetamol was investigated (**Figure 1.2**).

2. MATERIALS AND METHODS

2.1. Microorganisms and growth conditions

2.1.1. Batch cultures

An SRB consortium isolated from the wetland of the Urgeiriça mine (North of Portugal) containing mainly species affiliated to *Desulfovibrio desulfuricans* (Costa *et al*. 2012), was used as inoculum. These cultures were inoculated with 5% (v/v) inoculum and were grown and maintained in modified Postgate B medium: 0.5 g/L $K_3PO_4.H_2O$; 1 g/L NH_4Cl; 1 g/L $CaSO_4.2H_2O$; 1 g/L yeast extract; 2 g/L $MgSO_4.7H_2O$; 0.01 g/L resazurin; 0.5 g/L Na_2SO_3; 7.75 g/L $C_3H_5NaO_3$ in anaerobic conditions, at room temperature in 100 mL batch. The cultures were sub-cultured when almost all the sulphate was consumed, using 5% (v/v) of SRB inoculum (Palma *et al*. 2013).

SRB growth media samples were collected periodically from each batch with a syringe *via* the top of the glass flasks or from the bioreactor effluent, respectively.

Redox potential and pH of the growth media were measured using a pH/Eh Meter (GLP 21, Crison) to verify if the bacteria were growing in the optimal conditions. Sulphide concentration was measured immediately after sampling by the Methylene Blue Method (665 nm, Hach-Lange) and sulphate concentration was measured by the sulfaVer4 method (450 nm, Hach-Lange) using a UV-Visible spectrophotometer (DR 2800, Hach-Lange).

2.1.2. Lead sulphide particles and composites synthesis

The lead(II) solution was prepared by dissolving anidrous Lead(II) nitrate salt ($Pb(NO_3)_2$, 99.8%, VWR Prolabo) in ultra-pure water (milli-Q). Lead(II) concentration in the solution was about 100 mg/L.

TiO_2 of Degussa's P25 (*ca.* 80% anatase, 20% rutile), with an average primary particle size of ~21 nm was used as support for the synthesis of PbS nanocomposites.

The concentration of metals in the effluent of the bioremediation system and in the lead(II) solution before and after synthesis was determined by flame atomic absorption spectroscopy (Flame-AAS) using an Analytikjena NovAA 350 model spectrometer.

2.1.2.1. Synthesis using sulphide produced in batch culture

After evaluation of the SRB growth parameters, depending on the measured concentration of sulphide in the solution, an aliquot of the sulphide containing growth media was added to the lead ion solution described above. The addition was performed when over 90% of the sulphate had been converted to sulphide.

The growth culture volume added was calculated based on twice the sulphide content necessary to ensure the complete precipitation of the lead ions present in the solution. This addition, to 500 mL

of the metal ion solution, was carried out without filtration, in the absence or presence of 0.06 g TiO_2.

The growth medium with an excess of dissolved sulphide was added drop-by-drop, using a gas tight syringe with a hypodermic needle to the metal solution with or without the presence of TiO_2 under magnetic stirring at room temperature. After PbS or PbS/TiO_2 precipitation, the pH of the resulting suspension was measured, as well as the lead concentration remaining in the solution. The suspensions were centrifuged twice (Rotofix 32A, Hettich) for 10 minutes at 4000 rpm and washed twice with ethanol 70% (v/v) and dried under vacuum at room temperature (APT. Line VD, Binder). Assays were conducted in triplicate. A chemical synthesis using an aqueous solution of $Na_2S.9H_2O$ (Fluka, 32-38%) as precipitating agent was performed following the same steps than the biological synthesis (Palma *et al*. 2013).

2.2. Characterization of the synthesised material

The synthesized particles were analysed by X-Ray Powder Diffraction (XRD), using a PANalytical X'Pert Pro powder diffractometer, operating at 45 kV and 30 mA, with CuKα radiation filtered by Ni. XRD patterns were recorded using an X'Celerator detector, with a step size (2θ) of 0.0334 º, and a time per step of 499.745 seconds. Peak analysis and crystalline phase identification were conducted using the HighScore Plus software, with the ICDD PDF-2 database. The crystallite diameter of the synthesised particles (Dp) was estimated using the Scherrer equation (Scherrer, 1922), based on the form factor (K, 0.94) and considering the most intense peak observed for each crystalline structure present.

The specific surface area of the TiO_2 (Degussa P25) and PbS/TiO_2 was estimated following Brunauer, Emmett and Teller (B.E.T.) model. Nitrogen adsorption-desorption isotherm was measured on a NOVA Station A 2200e analyzer (Quantachrome Instrument Corporation, Boynton Beach, FL, USA) following the protocol as described in Ferreira *et al.* (2013).

Morphological and particle size characterization of precipitates was performed by Scanning Electron Microscopy (SEM) and by Transmission Electron Microscopy (TEM). SEM coupled with Energy-Dispersive X-ray System (EDS) analysis was carried out in a FEG-SEM (JEOL JSM7001F) instrument, at an accelerating voltage of 15 kV; the elemental analysis was carried out by EDS (Oxford XMax 150) with an accelerating voltage of 30 kV. The samples were prepared by deposition of the powder directly onto the carbon tape, which was then coated by carbon evaporation. TEM coupled with EDS was performed with a Hitachi 8100 with ThermoNoran light electron microscope at 160 kV of acceleration voltage. For the TEM measurements, a drop of the aqueous dispersion of PbS precipitates and respective TiO_2 composites was deposited on a copper

grid and dried at room temperature. The elemental analysis was carried out by semi-quantitative EDS.

Diffuse Reflectance Spectroscopy (DRS) was carried out in a UV-2600-Shimadzu UV/Vis spectrophotometer, using $BaSO_4$ (ReagentPlus, 99% - Sigma-Aldrich) as standard white board. The powder samples were placed in a sphere with a barium sulphate-coated inside and the reflectance percentage was measured from 200 to 1400 nm. The reflectance spectra were analyzed using the Kubelka-Munk (KM) function to convert the reflectance into the equivalent absorption coefficient (Reyes-Coronado *et al*. 2008). Thus, the formula $KM = \frac{(1-R)^2}{2R}$, where R (%) is the Reflectance/100 was used (**Equation 1**).

Photoluminescence of the dispersions was measured by a Fluorolog®-3/Horiba Jobin Yvon (Japan) with an excitation wavelength of 280 nm.

Zeta potential measurements of the "batch" synthesized PbS particles and PbS/TiO_2 composites, as well as for the used TiO_2 alone, were performed using a Malvern Nano ZS Zetasizer working at 146 V and 148 V, respectively. Each sample was suspended in aqueous solution, with pH values between 2 and 8 (adjusted with 5 M NaOH and/or HNO_3 65% solutions). After subjected to ultrasounds for 5 min these suspensions were then used for the zeta potential measurements in folded capillary cells.

2.3. Photodegradation studies

Photodegradation studies of CAP, an antibiotic considered as a model, and paracetamol the most commonly used drug worldwide with analgesic and antipyretic characteristics, were performed to investigate a possible application of the produced PbS nanocomposites.

The suspensions for the catalytic photodegradation studies for both drugs were prepared using 30 mg of PbS/TiO_2 nanocomposite or TiO_2 in 150 mL of aqueous solutions of CAP (10 mg/L) or paracetamol (20 mg/L). The photo decomposition reaction (photolysis) was performed with 150 mL of 10 mg/L and 20 mg/L CAP and paracetamol, respectively.

The particles were immersed in the aqueous solutions of CAP and paracetamol and prior irradiation, the suspensions were stirred in darkness for 60 min to ensure the adsorption equilibrium.

The initial value corresponds to the sample which was collected immediately after the adsorption and before irradiation. During light exposure the samples were collected at 5, 10, 20, 30, 45 and 60 minutes and over 240 and 265 min for photoreactor and sunlight exposition, respectively. All the samples were centrifuged (Rotofix 32A, Hettich) for 3 minutes at 3500 rpm. When TiO_2 was used alone as photocatalyst a second centrifugation (minispin, eppendorf) at 10000 rpm during 10 min was needed, whereas for the nanocomposites the centrifugation step, performed in the same

conditions, was done only once. The samples were analyzed by UV-Vis (UV-2600 Shimadzu) spectrophotometry in a spectrum range of 200 to 500 nm.

2.3.1. Photoreactor

Photolysis and photocatalysis of CAP was conducted in a refrigerated photoreactor with 250 mL of capacity. The solutions were irradiated by a 450 W medium-pressure mercury-vapour lamp from Hanovia where the total irradiated energy was 40 - 48% in the ultraviolet range and 40 - 43% in the visible region of the electromagnetic spectrum. During irradiation, suspensions were sampled at regular intervals over 60 min, centrifuged and analysed by an UV-2600 spectrophotometer purchased from Shimadzu Scientific Instruments (Japan) using the software UV Probe 2.42.

2.3.2. Sunlight light (UV and visible light irradiation) exposition

The photodegradation of CAP and paracetamol was investigated under sunlight exposition at environmental conditions. Photodegradation of paracetamol was only studied under natural sunlight exposition, because is the most advantageous process in terms of energetic costs performed under environmental conditions.

For the photodegradation experiments under sunlight irradiation the suitable UV conditions were based in the weather forecast IPMA (Portuguese Institute for Sea and Atmosphere) information. The experiments were performed with an UV index ranging from 7 to 9. During the irradiation assay, solutions were sampled at regular intervals along 240 min, centrifuged and monitored on a UV-VIS Pharo 300 spectroquant Merk Millipore molecular spectrophotometer at $\lambda_{máx}$ = 276 nm and 243 nm for CAP and paracetamol, respectively.

For both photoreactor and sunlight assays the photocatalytic efficiency (%) was determined using (**Equation 2**):

$$Photocatalytic\ efficiency\ (\%) = \frac{A_0 - A}{A_0} \times 100$$

where A_0 is the initial absorbance (when t = 0 min) of CAP and paracetamol and A is the absorbance of each drug during the photoirradiation assay, determined at $\lambda_{máx}$ of 276 nm and 243 nm for CAP and paracetamol, respectively.

3. Results and discussion

3.1. Biosynthesis of lead sulphide particles and composites by SRB batch cultures

For both biologically and chemically synthesis of lead sulphide particles and composites, an aqueous solution containing 101.4 mg/L of lead(II) with a pH of 5.47 and a redox potential of + 218 mV, was used. When the solution containing the biological generated sulphide was added to the lead(II) solution, in absence and presence of 0.06 g of TiO_2, the pH after precipitation was 6.79 and 6.81, respectively and the redox potential was -167 mV. The sulphide concentration in the culture growth medium was initially of 468.1 mg/L and after precipitation decreased to 8.1 mg/L (0.25 mM), which corresponds to a percentage of lead removal of about 100%.

When the chemical synthesis was performed using a $Na_2S.3H_2O$ aqueous solution as precipitating agent, the sulphide concentration was initially of 511.9 mg/L, decreasing to 14.9 mg/L (0.46 mM) after precipitation. The percentage of metal removal was about 90% and the redox potential of -189 mV. That lower percentage of lead removal obtained by chemical synthesis can be due to the high pH of the solution after precipitation (pH = 10.70), since for pH values above 8 the formation of metal hydroxy complexes is promoted and that can increase the metal solubility and reduce the precipitation effectiveness (Waller and Pickering, 1993; Palma *et al*. 2013).

The visual aspect of the synthesized PbS and PbS/TiO_2 particles using the sulphide generated by batch cultures matched the expected: a dark-grey solid, with metallic glow and a lighter shade of grey due to the presence of the white TiO_2.

One fact which had already been observed previously with other metals (unpublished author data), but which was particularly evident in the case of lead, was the formation of a layer of particles at the surface of the metal solution. These appeared as an apparently continuous film, with metallic glow. These particles were recovered with part of the solution (using a suction apparatus), separated in similar fashion (centrifugation and washing with 70% (v/v) ethanol) and characterized in the same manner as the particles synthesized. However, due to the small quantity recovered, the characterization of these particles was not as thorough as for the bulk precipitate (only XRD was performed).

3.2. Characterization of the obtained precipitates

Several techniques were used to characterize the chemical nature, morphology and particle size of the obtained precipitates, considering that those properties are important for eventual subsequent applications. Particularly in the present study the obtained particles were intended to be used as catalytic species in photodegradation reactions.

3.2.1. X-Ray Diffraction (XRD)

Figure 2.2 shows the XRD patterns of the synthesized precipitates and composites synthesized using sulphide generated in batch cultures.

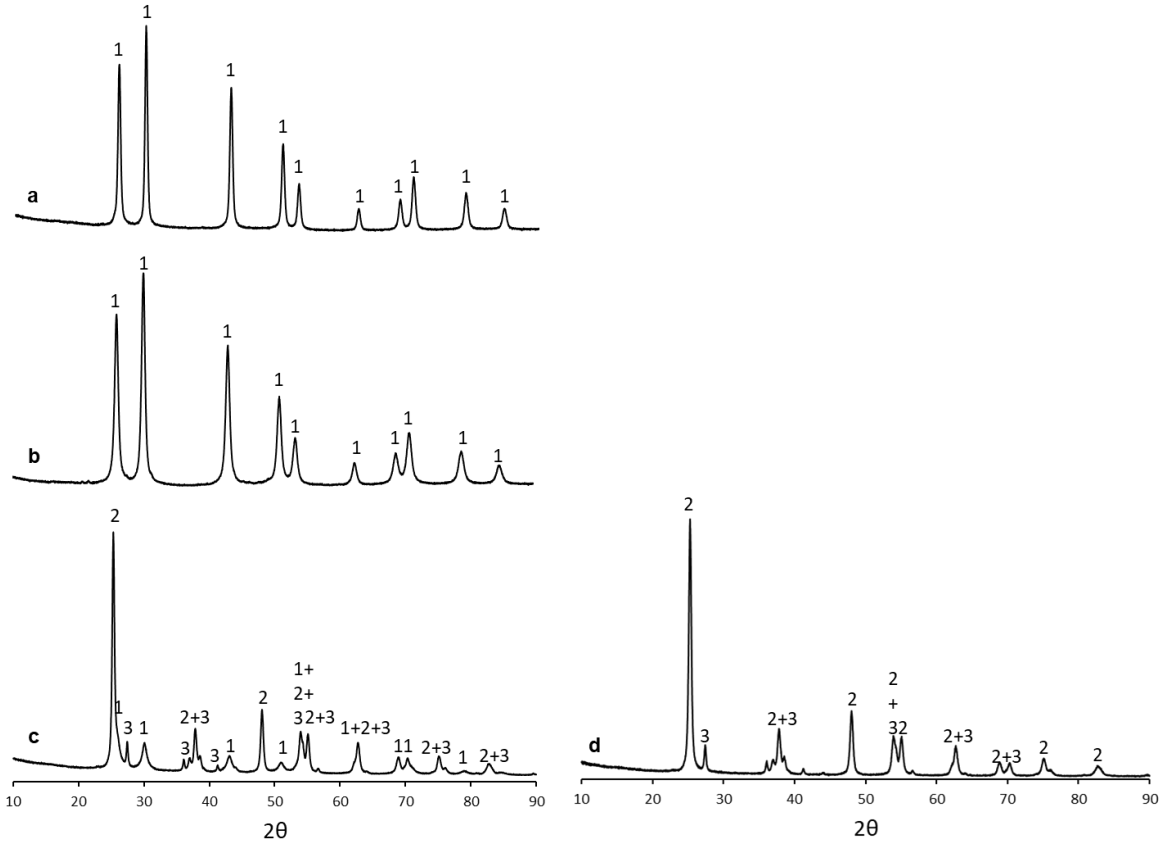


Figure 2.2 X-ray diffraction pattern of the precipitate obtained using (a) $Na_2S.9H_2O$ (chemical synthesis) and (b) biologically produced sulphide for the precipitation of dissolved Pb(II) and (c) PbS in association with TiO_2 (batch synthesis) and (d) the commercial TiO_2 used as support material. The peaks shown PbS (*galena*) "1", anatase "2", rutile "3"

The XRD results of **Figure 2.2a**, **b** show that the crystalline phase of the precipitates obtained are consistent with that of cubic PbS (*galena)*. According to the most intense peak observed at 30.24º and using the Scherrer equation and assuming Scherrer constant/shape factor of 0.94, the average PbS crystallite size was estimated as 25 nm. The diffractograms showed various diffraction peaks of PbS at 2θ values of 25.94º, 30.24º, 43.08º, 51.05º, 53.47º, 62.56º, 68.85º, 70.96º, 78.93º and 84.9º (**Figure 2.2a**, **b**). All peaks are in good agreement with the standard file (reference code: JCPDS#01-077-0244).

The diffractograms of PbS/TiO_2 samples in **Figure 2.2c** also show the same diffraction peaks of PbS that are consistent with that of PbS (*galena)* (reference code: JCPDS#01-077-0244). According to the most intense peak observed in **Figure 2.2c** and using the Scherrer equation, the average of the

PbS crystallite size in the nanocomposite sample was estimated as 20 nm. The crystallite size of the pristine TiO_2 was estimated as 20 nm, which agree with the manufacturer's information (TiO_2 particle size of ~21 nm).

As expected, from data supplied by the manufacturer Evonik Industries (2007) and previous studies (Costa *et al*. 2012; Vitor *et al*. 2015b), the used TiO_2 presented peaks characteristic of both anatase and rutile structures, which indicates that a mixture of both crystal phases were present.

Figure 2.3 presents the XRD patterns of the supernatant PbS recovered ("layer" produced during the recovery of the synthesised particles).

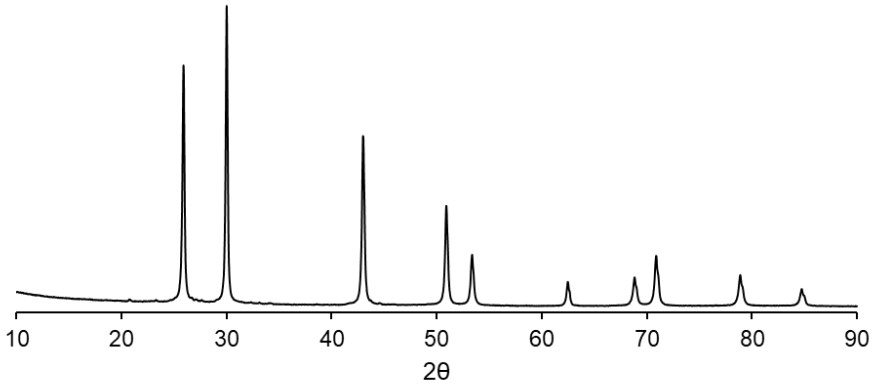


Figure 2.3 XRD patterns for supernatant of PbS synthesis

Analysing XRD data for bulk and supernatant PbS, it is evident that the patterns obtained were almost identical, indicating that the precipitates should be very similar (in fact, particle size estimated using the Scherrer equation indicated crystallite size around 50 nm. However, it is interesting to observe that the XRD diffractogram obtained for the supernatant did not include the extra peaks mentioned above, attributed to crystalline sulphur, thus indicating that these particles are purer (**Figure 2.3**).

The TiO_2 and PbS/TiO_2 powders were analysed by B.E.T. and adsorption isotherms of type II were obtained. The specific surface area of TiO_2 and PbS/TiO_2 estimated by B.E.T. isotherms model were 46.559 m^2/g and 38.005 m^2/g, respectively. The total pore volume of TiO_2 and PbS/TiO_2 were of 9.032×10^{-02} cm^3/g for pores smaller than 185.0 Å (Radius) and 8.64×10^{-02} cm^3/g for pores smaller than 187.5 Å (Radius), respectively. Thus, an evident reduction in the surface area of PbS/TiO_2 was observed, revealing that PbS suffered immobilization onto TiO_2 when synthesized using it as support.

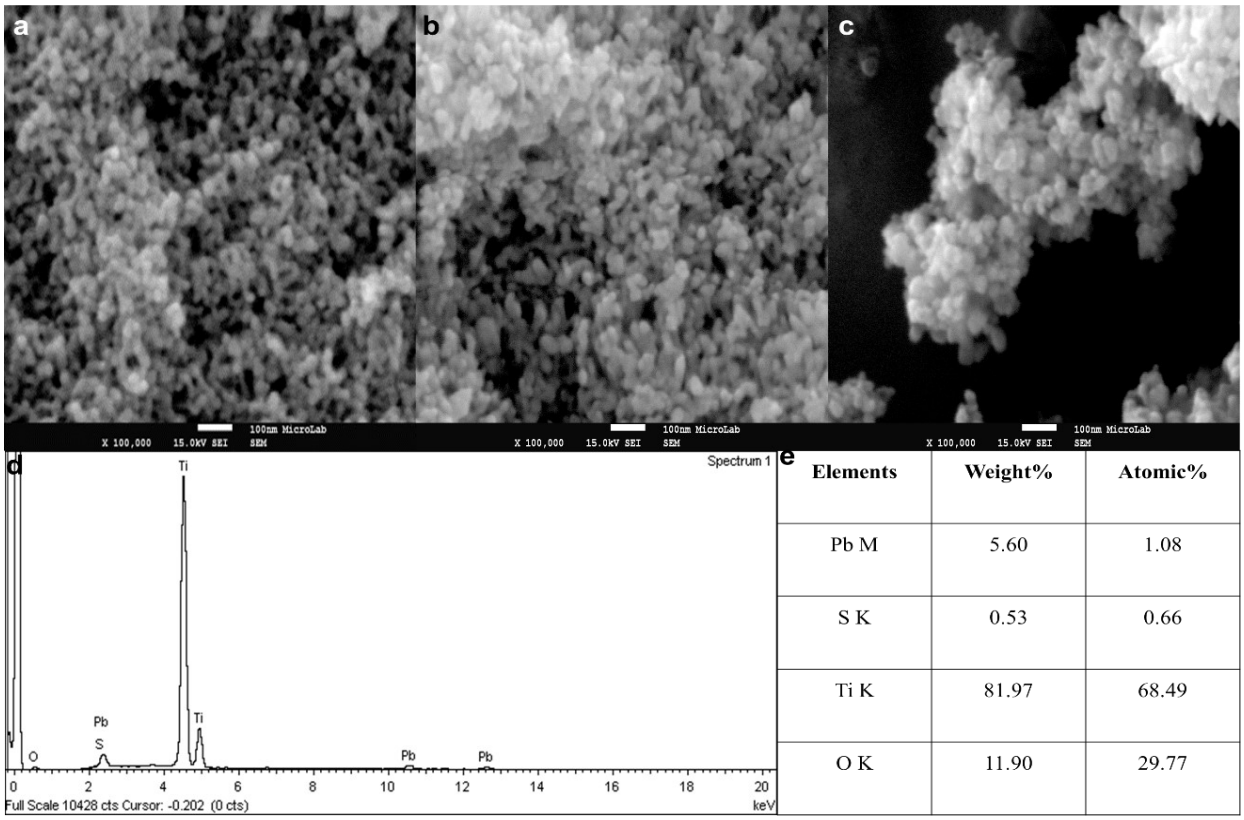
The values herein determined agree with those obtained by several authors such as Zhou and Huang (2012) and Ohno and colleagues (2001) who obtained for TiO_2 (Degussa, P25) a surface

area of 50 ± 15 m^2/g and 49.2 m^2/g, respectively. For example, Shkir *et al.* (2019) determined through B.E.T. analysis that PbS nanosheets presented a very low specific surface area of approximately 7 m^2/g. These results show that PbS/TiO_2 nanocomposites have a relatively large surface area with values close to those of TiO_2. These are generally important characteristics if a good heterogeneous catalyst with ability to adsorb the pollutants is desired (Ohno *et al.* 2001). However, some authors attest that there is no clear correlation between specific surface area and photocatalytic efficiency (Bumajdad *et al.* 2014; Vorontsov *et al.* 2018).

3.2.2. Electron Microscopy

3.2.2.1. Scanning Electron Microscopy - Energy-Dispersive X-Ray Spectroscopy (SEM-EDS)

SEM coupled to EDS provides important information about semiconductor samples including external morphology (texture), and chemical composition of materials which compose the sample. **Figure 2.4** shows SEM images of the synthesized nanoparticles and nanocomposites of PbS.



e

Elements	Weight%	Atomic%
Pb M	5.60	1.08
S K	0.53	0.66
Ti K	81.97	68.49
O K	11.90	29.77

Figure 2.4 SEM micrographs of PbS particles synthesized using sulphide generated: (a) chemically, (b) biologically (batch cultures), (c) biologically with TiO_2 as support (PbS/TiO_2) and EDS spectrum (d) with elements analysis (e) of representative of the composites

The PbS particles and composites, presented predominantly a spherical morphology (**Figure 2.4a, b, c**). UAPB system. The EDS elemental analyses (**Figure 2.4d**) of the composite that revealed the best photocatalytic characteristics, displayed peaks of Pb, S, Ti and O, suggesting a

successful synthesis of PbS coupled with TiO_2 as matrix. The calculated S atomic percentage of Pb:S was 1.08 and the obtained result from EDS is 0.66, thus confirming the formation of PbS nanoparticles in a ratio 1.63:1. The EDS also confirms the presence of TiO_2 in the composite (**Figure 2.4e**). The results of the EDS analysis showed that the atomic ratio of Ti:Pb was approximately 63:1. Due to the less relative quantity of PbS nanoparticles compared to those of TiO_2, they likely occupy a part of the surface of TiO_2 nanoparticles, leading that the rest of the support´s interface may be exposed.

3.2.2.2. Transmission Electron Microscopy - Energy-Dispersive X-Ray Spectroscopy (TEM-EDS)

TEM/EDS was performed to determine accurately characteristics of sample such as structure and morphology (*e.g.* size, shape).

Figure 2.5 shows a TEM image, a selected area electron diffraction pattern (SAED) and the EDS spectra of the synthesized PbS particles.

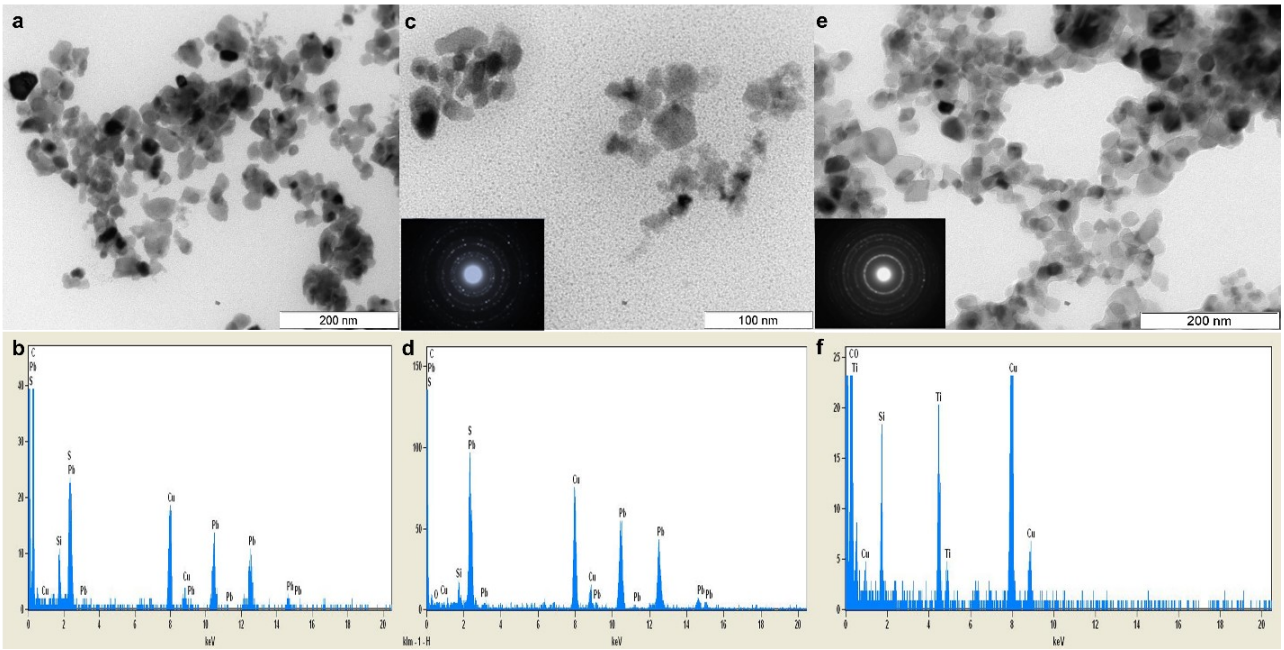


Figure 2.5 TEM micrographs of the PbS particles synthesized using the sulphide produced: (a) chemically, (c) in batch cultures and of (e) PbS/TiO_2 composites synthesized using sulphide generated in batch cultures and the corresponding EDS analysis b, d, f, respectively. (Insets SAED of the prepared powders)

As can be seen in **Figure 2.5a**, **c**, **e** the samples were homogeneous and the PbS particles present cubic and spherical shape with particle size ranging between 20 and 50 nm, in good accordance with the value estimated using the Scherrer equation. The SAED pattern (insets **Figure 2.5a**, **c**, **e**) confirms the crystallinity of the PbS nanoparticles. The EDS analysis (**Figure 2.5b**, **d** and **f**) allows

the identification of peaks assigned to Pb and S, which is consistent with the presence of PbS. Copper, also identified, was due to the utilization of a copper grid to support the sample.

The morphology of the PbS/TiO_2 sample, however, was not homogenous, as can be seen in **Figure 2.5e**. It can also be verified that some PbS was segregated from the ~20 nm TiO_2 nanoparticles, since the particles with cubic morphology corresponding to PbS can be perfectly distinguished from the particles with spherical morphology corresponding to TiO_2. The cubic particles presented sizes ranging between 20 and 50 nm, once again, these values are in good agreement with the values estimated by the Scherrer equation, confirming that the PbS was synthesized in the form of nanoparticles. The crystallinity of the particles was confirmed by SAED analysis.

As expected, the EDS spectra (**Figure 2.5f**) allowed the identification of Pb, S, Ti and O. The results demonstrated that no contamination of the precipitates by other elements occurred.

3.2.3. Zeta Potential

The zeta potential analysis allows the determination of the surface charge of nanoparticles and their agglomeration degree, thus being an indicator of stability (or lack thereof) in colloidal solutions (Mandzy *et al.* 2005; Jiang *et al.* 2009; Nimesh *et al.* 2017).

Nanoparticles have a charged surface that attracts to it ions of the opposite charge, a thin layer named as Stern layer, thus as nanoparticle diffuses throughout a solution this double layer of ions travels with it. Zeta potential is then the electric potential at the boundary of this double layer whose values typically vary from +100 mV to -100 mV, which also depends on the characteristics of the solution, namely of the pH (Mandzy *et al.* 2005; Jiang *et al.* 2009; Nimesh *et al.* 2017).

Nanoparticles, in a suspension, tend to suffer aggregation when their zeta potential values are greater than - 30 mV and less + 30 mV, due to Van der Waals, hydrophobic and hydrogen bond inter-particle attractions, while a high stability is usually displayed for zeta potential values outside this range (Mandzy *et al.* 2005; Jiang *et al.* 2009; Nimesh *et al.* 2017).

Figure 2.6 shows a plot of the measured zeta potential *versus* pH, for the TiO_2, PbS samples, and PbS/TiO_2 nanocomposite synthesized. Results obtained for TiO_2 are different to ones found in literature (Suttiponparnit *et al*. 2011; França *et al*. 2016), yet very close, and the difference can probably be attributed to the fact that samples were prepared in NaCl solution, whereas samples in the present study were prepared in ultrapure water.

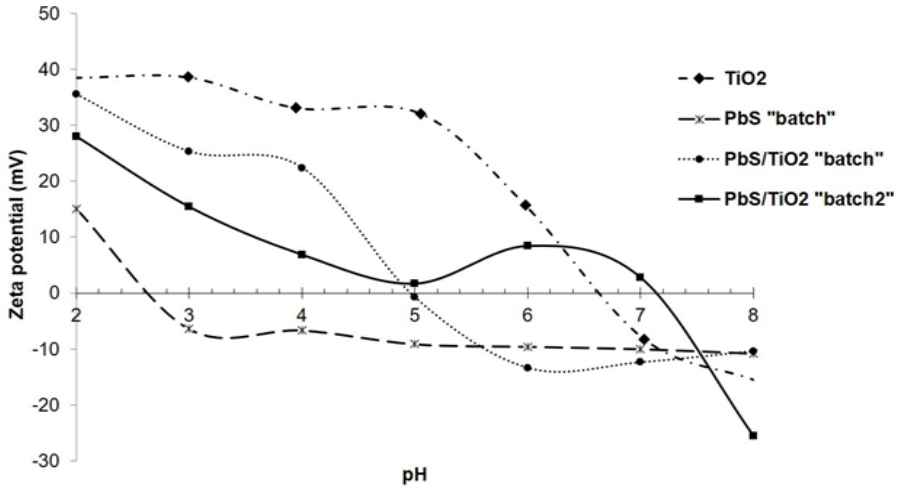


Figure 2.6 Plots of zeta potential as a function of pH, for PbS, TiO_2, and the PbS/TiO_2 nanocomposites

The results presented in **Figure 2.6** indicated that the PbS nanoparticles synthesized are susceptible of forming clusters, in the whole range of pH values tested, which can undoubtedly influence their performance as catalysts in photodegradation (Lin *et al.* 2013).

For the PbS particles, the zeta potential steadily decreased with the increasing of pH; for PbS synthesized using sulphide generated in batch, the isoelectric point (iep) is at pH 2.6, which is similar to values reported in the literature which are pH_{iep} 2.4 and pH 3 (Vergouw *et al*. 1998; Gaudin and Sun, 1996). Neville and Hunter (1976) observed that for well cleaned *galena* the iep is at pH_{iep} 2, and after prolonged conditioning the pH_{iep} increased to 4.

TiO_2 was also tested because it is well-known as a good photocatalyst on its own and it does not tend to form particle aggregates at pH values lower than about 4.6 - 5.5 (Mandzy *et al.* 2005; Jiang *et al.* 2009; Ren *et al.* 2017). The iep of the commercial TiO_2 occurs at pH_{iep} 6.6. However, the results obtained for the PbS/TiO_2 nanocomposite showed that the nanocomposite had much lower stability than pure TiO_2, since values of zeta potential between + 30mV and - 30 mV were obtained for all the pH range tested. Unlike what was observed for pristine PbS, the potential of the composite particles remained positive until pH 5, presenting the isoelectric point at this value, a little below than the value registered for the pure TiO_2.

3.2.4. UV-Vis Diffuse Reflectance Spectroscopy and Photoluminescence

The diffuse reflectance spectroscopy (DRS) is an important research tool used for materials optical characterization. An ideal photocatalyst should be characterized by the following attributes: photo-stability, chemically and biologically inert nature, availability and low cost and capability to adsorb reactant under efficient photonic activation (García *et al*. 2009). By DRS it is possible to conclude about the optical properties of semiconductor powder in the UV and visible range. The diffuse reflectance spectrum was recorded for the powders obtained by biologically generated sulphide.

Figure 2.7 shows the UV-Vis optical spectra of PbS nanoparticles and nanocomposites through the Kubelka-Munk function *vs* wavelength (nm).

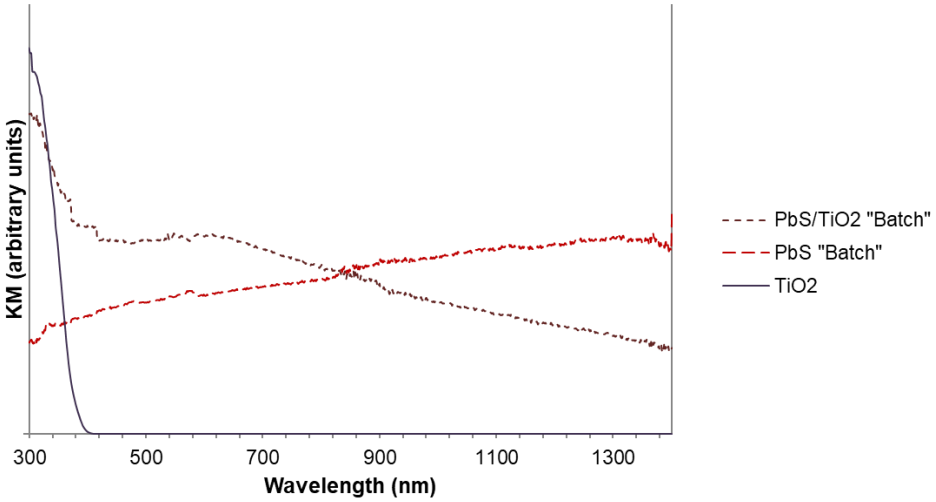


Figure 2.7 Optical spectra of PbS nanoparticles, PbS/TiO_2 nanocomposites produced by sulphide originated in batch and of commercial TiO_2 used as support material, in the UV-vis region

The absorption spectra of the PbS nanoparticles, PbS/TiO_2 nanocomposites and TiO_2 (**Figure 2.7**) were measured in order to evaluate their use as possible photocatalysts under visible light irradiation.

According to **Figure 2.7**, commercial TiO_2 presents a strong absorption edge around 390 nm (lower wavelengths) and a practically null absorption spectrum in the visible range (390-790 nm), which does not allow the utilization of visible light. Thus, TiO_2 can be activated only by UV radiation (320 – 400 nm) which corresponds to the UVA region. Due to this fact several past studies were focused on developing visible-light-sensitized photocatalysts, which consisted in coupling semiconductors of different bandgaps (Ola and Maroto-Valer, 2015; Guo *et al*. 2018). In this study PbS, a narrow bandgap semiconductor has been chosen to couple with TiO_2. According to **Figure 2.7**, the optical spectra of PbS (a black powder) exhibits a high absorption in the UV and visible regions. The spectra of PbS/TiO_2 "batch" powder shows that the addition of PbS to TiO_2 led to a clear red shift in the absorption band edge of the composite; such combination proved to be advantageous since the absorption wavelength of PbS/TiO_2 nanocomposites is extended to a visible region (higher wavelengths) due to the absorption of visible light by PbS (**Figure 2.7**). PbS/TiO_2 "Batch" powder exhibited the highest irradiation absorption in the visible range of all the tested materials (**Figure 2.7**). Thus, from the combination of TiO_2 particles with other semiconductor phase, midgap states can be generated where direct recombination occurs, producing composites with high visible light photocatalytic activity. Therefore, the semiconductor with lower bandgap absorbs the photons from visible light and the resulting photogenerated electrons are subsequently transferred to the semiconductor with larger bandgap energy (Angel *et al.* 2018; Kumar and Devi, 2011; Wu *et al.*

2006). As a direct consequence of this synergetic combination photocatalysts with high performance can be prepared (Palma *et al*. 2013).

The optical bandgap energies were calculated using the procedure described by Entradas and co-workers (2014). However, it was only possible to determine accurately the bandgap energy for TiO_2, which was about 3.1 eV, value that is in accordance with the obtained by Kim and Kim (2012). In the case of PbS nanoparticles and for PbS/TiO_2 nanocomposites it was not possible to determine the bandgap with precision since no well-defined band absorption edges were observed due to its near continuous absorption in the UV-vis region.

Figure 2.8 shows the photoluminescence spectra of PbS nanoparticles and nanocomposites through fluorescence *vs* wavelength (nm).

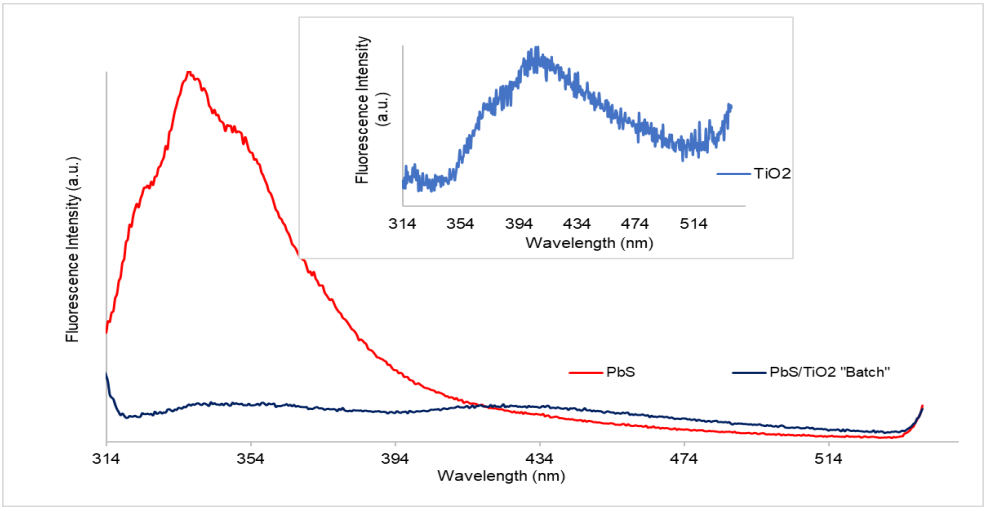


Figure 2.8 Photoluminescence emission spectra of PbS and TiO_2 nanoparticles and of PbS/TiO_2 nanocomposites with an excitation wavelength of 280 nm

A decrease in the fluorescence emission was observed when PbS particles were grown in the presence of TiO_2 nanoparticles **(Figure 2.8)**. This decrease is more pronounced when the nanocomposite is prepared using the batch process. This behavior has been associated with a decrease in the photo-generated electron-hole recombination rate, due to a more efficient electron-hole separation. As a consequence, the photocatalytic performance of such composite materials is expected to be enhanced when compared to pristine PbS and TiO_2 nanoparticles. The association of PbS/TiO_2 is capable of absorbing radiation in an extended spectral range, from the UV to visible range of sunlight, thus enhancing the photocatalytic activity. This reinforces the interest in the use of these materials in photocatalytic reactions, since they have transitions in the visible range, in addition to those in the UV region of the spectrum.

3.3. Photodegradation studies

Photodegradation studies of CAP, an antibiotic considered as a model, and of paracetamol an analgesic drug widely used, were performed as possible applications for the produced PbS nanocomposites.

Photolysis (without photocatalyst) was performed in a photo-reactor and under sunlight irradiation (solar exposition) with an initial solution of 10 mg/L and 20 mg/L of CAP and paracetamol, respectively.

Since some pollutants can be degraded in the presence of UV light, they must absorb the light with a reasonable photo-dissociation quantum yield which can be defined as the number of pollutant molecules degraded in a reaction over the number of photons absorbed (Ishibashi *et al*. 2000; Lasa *et al.* 2005). Although organic pollutants absorb light over a wide range of wavelengths, this absorption is generally stronger at lower wavelengths. Thus, photolysis experiments must be performed always before photocatalysis tests in order to find out decomposition rates without catalyst. In the photochemical processes, solar photons are directly absorbed by reactants and/or a catalyst causing a reaction. This path leads to a chemical reaction produced by the energy of the sunlight's photons (Gálvez and Rodríguez, 2010).

The selection of an optimal catalyst loading is essential to avoid excesses that limits the amount of light reaching, thus blocking the photodegradation, and to ensure a total absorption of efficient photons. The photocatalytic characteristics of PbS, considering that all the Pb(II) was precipitated in association with different amounts of TiO_2 (0.04 g, 0.06 g and 0.08 g) were previously studied (Palma *et al*. 2013). Based on the previous results the composite which displayed the optimal photocatalytic features was chosen to perform the photodegradation experiments. Thus, each photodegradation assay was performed using an initial solution of 10 mg/L of CAP and 20 mg/L of paracetamol in the presence of 30 mg of TiO_2 or PbS/0.06 g TiO_2 that were used as photocatalysts.

The pH of the initial suspensions of CAP and paracetamol were 5.89 and 6.55 and the final pH values were 4.27 and 5.34, respectively.

CAP did not present an isoelectric point, and as it remained negatively charged at all studied pHs. That fact enabled the interactions between the drug and the nanocomposite at pH 5.89, since at that pH the PbS/TiO_2 nanocomposite synthesized is positively charged. However, PbS cannot be used alone as CAP photocatalyst since it remained always negatively charged.

According to literature, the pKa of paracetamol is 9.38 (Bernal *et al*. 2017). Thus, at lower pHs the drug mostly exists as a neutral charged molecule and as it approximates to its pKa about half of paracetamol is ionized and become anionic behaviour already described for phenolic drugs (Ferreira *et al.* 2015; Mohd *et al.* 2015). The pH also influences the charges that may appear in the nanocomposite surface (**Figure 2.6**). Moreover, it should be noted that at pH 6.80 paracetamol is

neutrally charged (pKa *ca.* 9.5) (França *et al*. 2016). Thus, at pH 6.55 paracetamol exibhits neutral charge and although neither attraction nor repulsion is favored, the drug can be adsorbed by the equally neutral and positively or negatively charged nanocomposites surface. For example, França and colleagues (2016) observed that the higher adsorption, between paracetamol and catalyst (TiO_2 and 2.5 wt.% of Zn(II) phthalocyanine (TiO_2/ZnPc)), occurred at the isoelectric point measured for the nanocomposite.

3.3.1. Photodegradation of CAP in a photoreactor

The photolysis and photocatalytic degradation of an aqueous solution of 10 mg/L CAP were studied. Photodegradation was performed in the presence of TiO_2 nanoparticles and PbS/TiO_2 nanocomposite as catalysts, in the photoreactor (450 W) with an irradiation time of 60 min (**Figure 2.9**).

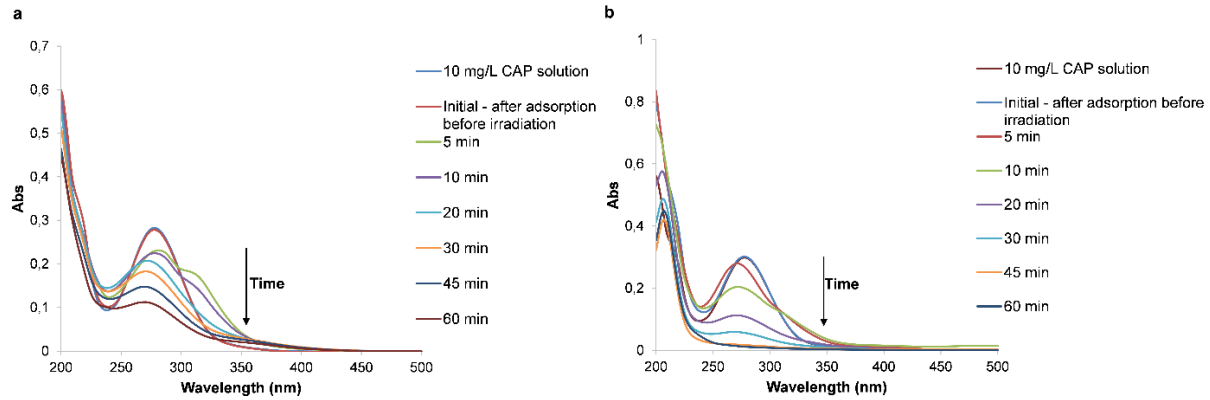


Figure 2.9 UV-vis absorption spectra of a 10 mg/L chloramphenicol solution during 60 min of photolysis (a) and using PbS/TiO_2 nanocomposite as photocatalyst (b), in the photoreactor (450 W)

The absorption spectra show that the antibiotic CAP suffered some degradation in the absence of catalyst (photolysis), but it was not completely degraded, at the described conditions along the 60 min of irradiation (**Figure 2.9a**). When the nanocomposites were used as photocatalysts as it was expected the photodegradation of the antibiotic were faster and complete (**Figure 2.9b**).

The UV absorption spectra of 10 mg/L CAP photodegradation revealed that when TiO_2 (positive reference) was used as photocatalyst a faster and almost complete photodegradation of the antibiotic was achieved after 60 min of irradiation.

The CAP degradation profiles are presented in **Figure 2.10**.

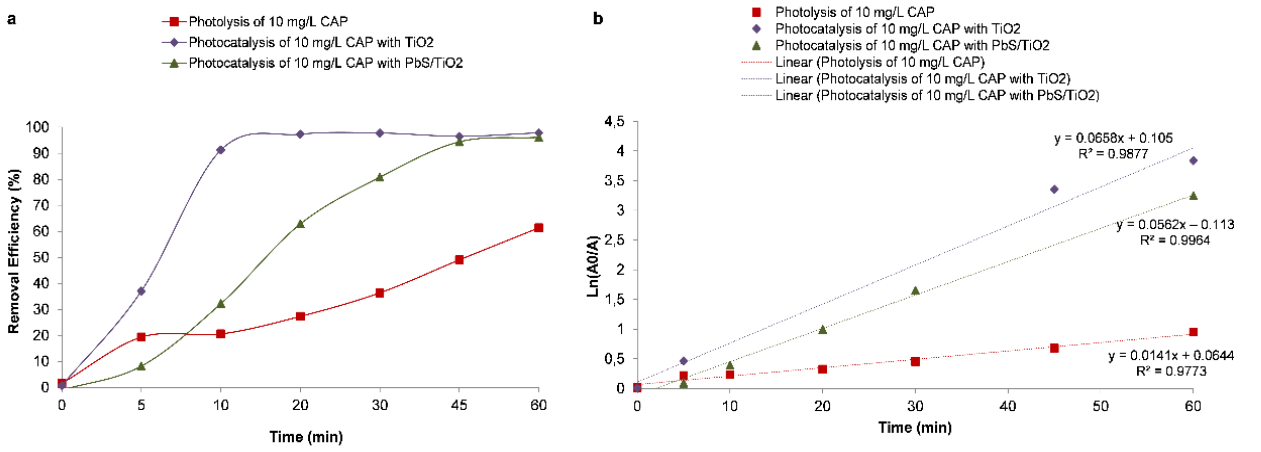


Figure 2.10 Degradation efficiency (%) of 10 mg/L solution of CAP during photolysis and using PbS/TiO_2 nanocomposite and TiO_2 as photocatalysts *versus* irradiation time, during 60 min (a). Kinetics of the CAP photodegradation reaction *via* photolysis and using TiO_2 and PbS/TiO_2 as photocatalysts (b)

By analyzing **Figure 2.10a** it is possible to conclude that CAP did not suffer a complete photolysis after 60 min of irradiation, whereas using TiO_2 and PbS/TiO_2 nanocomposite as photocatalysts the degradation was almost complete. The removal of CAP by photolysis was about 61% after 60 min of assay, while when using TiO_2 as catalyst the removal of the drug was about 91% and 98% after 10 min and 30 min assay, respectively. The use of PbS/TiO_2 nanocomposite as photocatalyst showed slower photocatalytic removal compared to TiO_2, but after 45 min the removal was almost complete (98%). The PbS/TiO_2 nanocomposite presented a very similar activity to TiO_2 after 45 min of reaction (**Figure 2.10a**), which suggests that it may be an alternative photocatalyst to TiO_2 for CAP degradation. It should be noted that the nanocomposite can be synthesized economically as a consequence of a bioremediation process for AMD treatment and the reduced amount of TiO_2 used may be seen as an advantage.

Various photocatalytic processes have been described by different kinetic models, such as pseudo zero order, pseudo first order and second order models. Assuming that the photocatalytic reactions could be fit to a pseudo-first-order degradation that follows the kinetic model of Langmuir–Hinshelwood (L–H) the expression $ln\frac{A_0}{A} = K_{app} \times t$ (**Equation 3**) is applied. A_0 corresponds to initial absorbance and A to absorbance at time t during the course of reaction, K_{app} is the slope of the linear regression corresponding to apparent degradation rate, which is considered the response and t is the irradiation time. Thus, if the correlation coefficient (R^2) from the linear fit is greater than 0.95 then it follows the pseudo-first order kinetics (Ho 2004).

The estimation of half-lives, that is defined as the time required for decay from the initial value to 50%, was as on the formula $t_{1/2} = \frac{0.693}{K_{app}}$ (**Equation 4**), where K_{app} is the estimated pseudo-first-order degradation rate constant (Ho 2004; Gaya 2014).

Experimental data agreed with the L–H model due to the linear behavior of each curve which indicates that a pseudo first order reaction was occurring during CAP degradation (**Figure 2.10b**). According to the kinetics parameters, the photolysis presented the lowest K_{app} value of 0.0141 min^{-1}. The best photocatalytic activity and consequently faster degradation was observed for TiO_2, that exhibited the highest K_{app} of 0.0658 min^{-1} (**Figure 2.10b**), whereas for the tested nanocomposite PbS/TiO_2 the K_{app} was of 0.0562 min^{-1}. The estimation of half-lives of each reaction was of 10.53, 12.33 and 49.15 min for TiO_2, PbS/TiO_2 and photolysis, respectively.

3.3.2. Sunligth and UV irradiation

In the photocatalysis under solar light irradiation and operated at environmental conditions the time of reaction surely increases due to fluctuations in solar intensity possibly leading to a slower reaction, while photoreactors are specially designed to use artificial illumination allowing more efficient and intensive processes. The use of artificial light also presents some disadvantages considering that the production of photons typically dominates the annual operating costs of photocatalytic processes. Thus, the use of solar irradiation in addition to be a low-cost process also reduces the carbon footprint of the system (Braham and Harris, 2009).

3.3.2.1. Chloramphenicol

Aqueous solutions of 10 mg/L CAP, both in presence of TiO_2 and PbS/TiO_2 nanocomposite as catalysts, were irradiated by sunlight at UV index ranging from 7 to 9 and under environmental conditions. The photolysis and photocatalytic degradation of CAP were studied, and the results are shown in **Figure 2.11**.

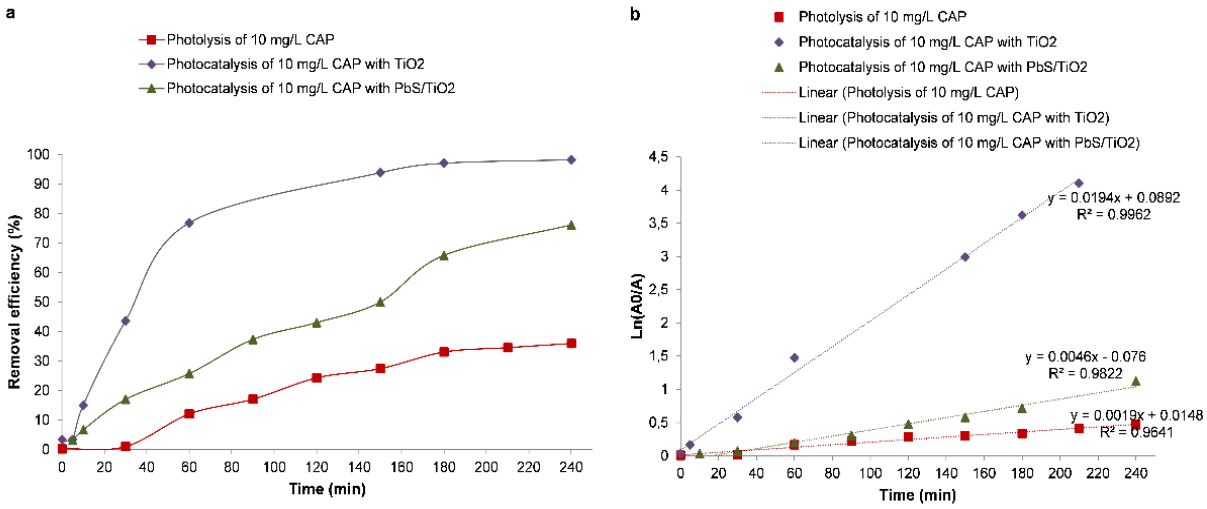


Figure 2.11 Degradation efficiency (%) of 10 mg/L solution of CAP during photolysis and using PbS/TiO_2 nanocomposite and TiO_2 as photocatalysts *versus* solar exposure time (a) at UV index ranging from 7 to 9. Kinetics of the CAP photodegradation reaction *via* photolysis and using TiO_2 and PbS/TiO_2 as a photocatalysts (b)

The photolysis degradation efficiency of 10 mg/L CAP under solar ligth was about 36% after 240 min. In the photodegradation of CAP using TiO_2 as photocatalyst, the removal efficiency was about 97% and 98% after 180 min and 240 min, respectively, whereas the photodegradation of 10 mg/L of CAP by the PbS/TiO_2 nanocomposite showed a removal efficiency of 63% and 76%, after 180 and 240 min of irradiation, respectively (**Figure 2.11 a**).

In the case of sunlight photodegradation studies, experimental data agree with the L–H model due to the linear behavior of each curve with correlation coefficients (R^2) higher than 0.95 (**Figure 2.11b**).

According to the kinetics parameters, the photolysis presented the lowest K_{app} value of 0.0019 min^{-1}. The faster photodegradation activity was observed for TiO_2, that exhibited the highest K_{app} of 0.0194 min^{-1}, while using the nanocomposite PbS/TiO_2 the K_{app} was of 0.0046 min^{-1} for 10 mg/L CAP (**Figure 2.11b**).

The half-life of each reaction was estimated as 35.72, 150.65 and 364.74 min for TiO_2, PbS/TiO_2 and photolysis, respectively.

Trovó and colleagues (2014) evaluated the photodegradation of CAP in the absence of catalyst (photolysis) in ultrapure water, untreated surface water, and treated effluent from sewage treatment plant in laboratory scale and pilot scale, using solar and artificial radiation. The results revealed that CAP transformation was strongly influenced by the radiation source. A higher dose of UVA per unit of volume in the solar experiments was necessary to reach the same results obtained using artificial radiation due to the higher absorption of photons by CAP inside the spectral distribution of the radiation furnished by a 400 W high pressure mercury lamp, between 295 and 800 nm, when compared to the solar spectral distribution in this same spectral range (Trovó *et al*. 2014). The same authors showed that after 180 and 1440 min of artificial and solar irradiation 99.2 and 97.7% of CAP degradation occurred by photolysis. Our experiment did not last so long, but the trend of a slowly CAP degradation seems to be the same. The CAP photolysis was about 61% after 60 min irradiation in the photoreactor and of 36% after 240 min of Sunlight exposition. Trovó and colleagues (2014) also demonstrated that the rates of CAP degradation were better fitted considering a pseudo-first order rate law for both radiation sources and matrices studied. The k_{app} and $t_{1/2}$ were strongly influenced by the radiation source, as it was observed in the present work.

To the best of our knowledge there are no results in the literature about CAP photocatalysis using PbS/TiO_2 as catalyst.

3.3.2.2. Paracetamol

The photocatalytic degradation of paracetamol was studied, and the results are presented in **Figure 2.12**.

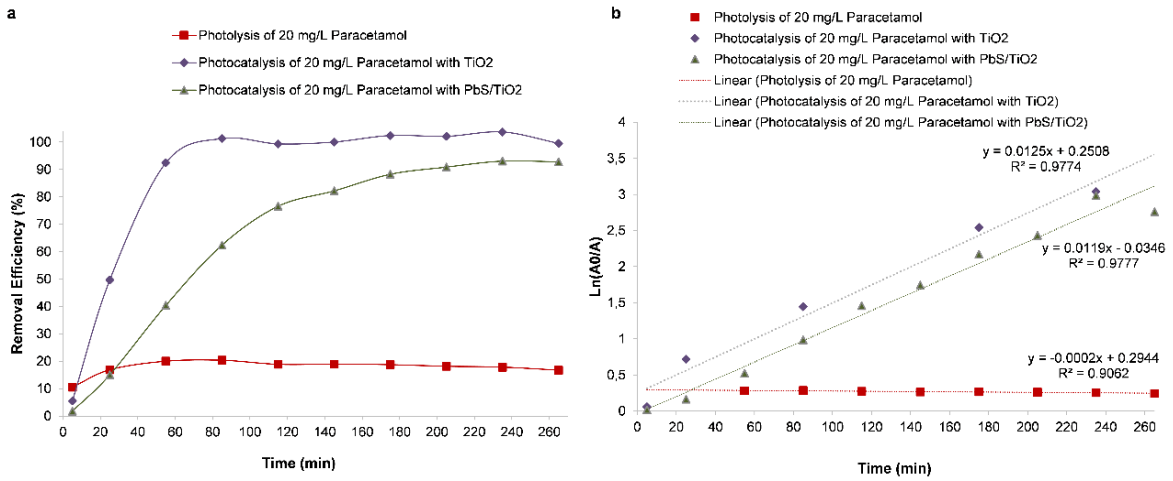


Figure 2.12 Degradation efficiency (%) of 20 mg/L solution of paracetamol during photolysis and using PbS/TiO_2 nanocomposite and TiO_2 as photocatalysts *versus* solar exposure time (a) at UV index ranging from 7 to 9. Kinetics of the paracetamol photodegradation reaction *via* photolysis and using TiO_2 and PbS/TiO_2 as a photocatalysts (b)

Several research groups studied the photocatalytic degradation of paracetamol solutions using TiO_2 and UV-light. The results indicated that paracetamol is not mineralized by UV-light in the absence of catalyst; the degradation of the drug is less than 10%. However, in the presence of the optimal amount of TiO_2 the reaction rate and the overall conversion increases (Yang *et al*. 2008; Aguilar *et al*. 2011; Desale *et al*. 2013). Karaman and co-workers (2016) investigated the degradation of 80 ppb paracetamol by UVA/TiO_2 experiments under neutral conditions. It took 20 min for paracetamol to degrade. Those results corroborate the ones obtained in the present study, where the removal of paracetamol by photolysis was about 18% after 265 min of assay, while when TiO_2 was used as photocatalyst the removal of the drug was about 99% after 115 min of irradiation (**Figure 2.12a**). The use of PbS/TiO_2 nanocomposite as catalyst did not show a faster photocatalytic removal compared to pristine TiO_2. In the presence of the nanocomposite paracetamol removal achieved 82%, after 145 min of irradiation. Nevertheless, after 235 min of sunlight irradiation the removal efficiency of paracetamol got closer to that one obtained by TiO_2, achieving a value of 93% (**Figure 2.12a**), efficiency which has remained constant until the end of the experiment (265 min).

The experimental data from 20 mg/L paracetamol photocatalysis agree with the L–H model, since a plot of $\ln(A/A_0)$ *versus* irradiation time resulted in a straight line whose slope is the k_{app}, and the regression analysis usually resulted in correlation coefficients (R^2) higher than 0.95 (**Figure 2.12b**). The degradation process of paracetamol described by Aguilar and colleagues (2011) also followed a pseudo first order degradation behavior, which was represented by the Langmuir-Hinshelwood equation. However, the photolysis of paracetamol seems to follow a pseudo zero order reaction, since the reaction rate is independent of concentration of the drug. In this case the limiting factor

could be something as the absorption of light in the photolysis reaction. The half-life of zero order reaction is given by $t_{1/2} = \frac{A0}{2K_{app}}$ (**Equation 5**).

According to the kinetics parameters, the photolysis presented the lowest K_{app} value of 0.0002 min^{-1}. Once again, the faster photocatalytic activity was observed for TiO_2, that exhibited the highest K_{app} of 0.0125 min^{-1}, whereas using the nanocomposite PbS/TiO_2 the K_{app} was of 0.0119 min^{-1} for 20 mg/L paracetamol (**Figure 2.11b**).

The estimation of half-lives of each reaction was of 55.44, 58.24 and 4238.75 min for TiO_2, PbS/TiO_2 and photolysis, respectively.

Desale and colleagues (2013) found that for small concentrations of paracetamol up to 100 mg/L, the degradation was faster, and the total conversion takes place after 240 min, which also corroborates the results herein described. On the contrary, when the concentration is high, the degradation requires more time, and the kinetic reaction adjusts to a zero-order reaction rate equation (Aguilar *et al*. 2011; Desale *et al*. 2013).

It should be noted that the photodegradation of paracetamol occurs more rapidly than biodegradation (Desale *et al*. 2013). Paracetamol photodegradation in the presence of a chemically inert compound such as TiO_2 or a composite (PbS/TiO_2) under sunlight irradiation took about 4 h to be completely degraded (Desale *et al*. 2013), while the degradation of paracetamol from municipal wastewater in the presence of aerobic bacteria, was 99.9% after 2 days incubation with aeration (Palma *et al*. 2018).

For CAP and paracetamol photocatalysis assays performed in photoreactor and under sunlight irradiation in the presence of the PbS/TiO_2 nanocomposites, the photodegradation of both drugs was slower than in the presence of TiO_2 and that may be due to the presence of impurities since these nanocomposites are synthesized during a biological process. Such impurities can be bacterial debris, which can limit the amount of light reaching the reactional species, thus blocking the photodegradation. Other factors may have influence in the optimal characteristics of catalysts, such as the occurrence of segregation of PbS from the TiO_2, which was observed in certain samples, as demonstrated by TEM analysis (**Figure 2.5**) and the formation PbS/TiO_2 clusters during photodegradation, since those experiments occurred at pH about 6, where the nanocomposite presented less stability (**Figure 2.6**). In addition to that, the pH can also affect the adsorption of the pollutants to the catalyst and consequently the photodegradation. Zhang and colleagues (2010) related that for CAP photodegradation with TiO_2, the optimum pH was of 6.4, value that is near to the one herein described in CAP experiments (pH 5.8). They also found that in the presence of TiO_2, the CAP degradation rate slowly increased between pH 3.5 and 9.5, but significantly decreased with increasing pH between 9.5 and 11.0 (Zhang and colleagues, 2010). Yang *et al.*

(2008) and Jallouli and co-workers (2014) found that pH 9.0 was the optimum for paracetamol photodegradation.

Although photocatalysis using PbS/TiO_2 nanocomposites as catalyst was not so efficient as TiO_2 regarding the degradation of the drugs under study, these nanocomposites become attractive both economically and environmentally, due to the fact that they can be synthesized from an AMD bioremediation process with SRB, in which the treated effluent containing sulphide in excess can be used to precipitate the metals present in effluents from metallurgical industry.

4. CONCLUSIONS

PbS nanoparticles and PbS/TiO_2 nanocomposites were successfully synthesized, using biological sulphide produced by SRB in batch cultures. PbS/TiO_2 nanocomposites were used as photocatalysts in the degradation of CAP and paracetamol, since they displayed the best characteristics aiming their use as potential catalyst.

TEM analysis showed that PbS nanoparticles and PbS/TiO_2 composites obtained using sulphide generated in batch cultures have an average particle size ranging from 17 to 25 nm and 15 to 20 nm, respectively, with cubic structure, while EDS analysis confirmed the presence of lead and sulphur in the PbS particles.

XRD studies indicate that batch synthesized particles are of cubic *galena*. The synthesised particles have proven to be of a relatively high degree of purity, despite the presence of excess sulphur identified in the XRD patterns. Zeta potential studies indicate that both PbS and the corresponding TiO_2 nanocomposite tend to form clusters at pH 2 to 8.

The obtained results related to photolysis and photocatalytic degradation in the tested conditions indicated that CAP and paracetamol were not completely degraded in the absence of catalyst (photolysis). Nevertheless, both drugs can be completely degraded in the presence of a catalyst in photoreactor at 450 W and by sunlight exposition (UV and visible light). The presence of the nanocomposite PbS/TiO_2 and TiO_2 increases the reaction rate and the overall conversion. The degradation process follows a pseudo first order degradation.

The photodegradation of CAP by photolysis was of about 61% and 36%, after 60 min irradiation at 450 W and under sunlight exposition, respectively. In the presence of nanocomposite as catalyst, a high drug removal efficiency of about 96% and 93% was achieved after 60 min and 240 min irradiation, respectively, while using TiO_2 the removal efficiency was about 98% for both photoreactor and sunlight irradiation.

Paracetamol photocatalysis using PbS/TiO_2 nanocomposites was also successfully performed under sunlight exposition with a high removal of 93%, while paracetamol photodegradation using TiO_2

was complete (about 100%), after 265 min of irradiation. Drug's removal by photolysis was about 18%.

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CHAPTER 3

Putative role of *Flavobacterium*, *Dokdonella* and *Methylophilus* strains in paracetamol biodegradation

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ABSTRACT

Paracetamol, the most widely and globally used analgesic and antipyretic, is easily accumulated in aquatic environments. In the present study, the biodegradation of paracetamol in different media (one for general growth, one specific for sulphate reducing bacteria, a mineral salt medium and municipal wastewater) inoculated with two types of sludge (from anaerobic lagoon and from oxidation ditch) under different oxygenic conditions (anoxic; moderate oxygenation in open flasks and high oxygenation by aeration) was investigated. In addition, bacteria with relative abundances increasing simultaneously with paracetamol degradation, when this drug was the only carbon source, thus with a putative role in its degradation, were identified using 16S rRNA gene sequences. The results show that aerobic microorganisms had a major role in the degradation of paracetamol, with 50 mg/L totally removed from municipal wastewater after two days incubation with aeration, and that the metabolites 4-aminophenol and hydroquinone plus one compound not identified in this work were produced in the process. The identification of bacteria with a role in the degradation of paracetamol revealed a strain from genus *Pseudomonas* with the highest final relative abundance of 21.2%, confirming previous works reporting strains of this genus as paracetamol decomposers. Besides, genera *Flavobacterium*, *Dokdonella* and *Methylophilus* were also in evidence, with initial relative abundances of 1.66%, 1.48% and 0.00% (not detected) in the inoculum and 6.91%, 3.80% and 3.83% after incubation, respectively. Therefore, a putative role of these genera in paracetamol biodegradation is suggested for the first time.

Keywords: Acetaminophen; 4-aminophenol; Bioremediation; Hydroquinone; Pharmaceutical compounds

1. INTRODUCTION

Emerging pollutants are not covered by existing water quality regulations, but they are considered potential threats to environmental ecosystems, human health and safety, especially because their large-scale production and use results in their release into the environment usually via sewage and wastewater treatment plant (WWTP) discharges (Deblonde *et al*. 2011). The biodegradation and behavior of these compounds in aqueous systems are largely unknown, leading to simplified assumptions in the estimation of their environmental risk. Additionally, they may have an impact on wastewater treatment processes by increasing or decreasing sewage or industrial contaminant removal efficiency (Deblonde *et al*. 2011).

Paracetamol (N-(4-hydroxyphenyl)acetamide), or acetaminophen, is the most consumed analgesic. For example, in France 3303.08 tonnes were consumed in year 2008 (Vulliet and Cren-Olivé, 2011). It is considered an emerging pollutant due to its widespread global use and to the fact that it is readily accumulated in aquatic ecosystems with adverse effects (De Gusseme *et al*. 2011; Wu *et al*. 2012; Mbokou *et al*. 2016). Though paracetamol appears to be well removed during sewage treatment when compared to other pharmaceuticals, its concentrations detected in surface waters ranges from 110 to 10000 ng/L (Wilkinson *et al*. 2017). A warning sign of the global importance of this pollutant is the detection of paracetamol in the open sea waters of the western Mediterranean in concentration ranges from 0.468 to 1.70 ng/L (Brumovský *et al*. 2017). In fact, the worldwide presence of this drug in the WWTP effluents reminds the need of implementing treatment processes more efficient for its removal, even if in general the higher concentrations of paracetamol detected in raw sewage than in treated wastewaters indicate high removal rates. For example, concentrations up to 180 µg/L have been reported in WWTP influents while in the effluents the concentrations reported were up to 0.305 µg/L in systems with aerobic tanks and up to 13 µg/L in wetlands (*e.g.* Roberts and Thomas, 2006; Papageorgiou *et al*. 2016; Vymazal *et al*. 2017). In Portugal, the highest concentration of paracetamol recorded in wastewater effluent was 32 µg/L (Pereira *et al*. 2016). Moreover, paracetamol´s main metabolite from animals, paracetamol glucuronide, also enters the environment via human excretion and has been detected in WWTP effluents at levels of up to 462 µg/L (Santos *et al*. 2013). Paracetamol glucuronide has been detected in Portuguese surface waters at concentrations of up to 3.57 µg/L (Santos *et al*. 2013).

Although paracetamol stability decreases in acidic or alkaline conditions due to be slowly degraded via a base- or acid-catalyzed hydrolysis of the amide bond into acetic acid and 4-aminophenol, stability studies in purified water at room temperature demonstrated that after incubation for more than one month this drug was completely stable due to the high energy needed to overcome the barrier for the cleavage of its amide bond (Karaman *et al*. 2016). In addition, being 4-aminophenol

a photo and thermal sensitive compound, like other aromatic amines, the results obtained by Khan *et al.* (2006) showed the occurrence of its autoxidation after 7 days but at low rates: 0.8%, 26.6%, 20% and 13% at 50, 100, 150 and 200 mg/L, respectively. In any case, the authors of both works concluded that biodegradation was more effective. Indeed, several studies have demonstrated that microorganisms play a major role in the environmental degradation of paracetamol and during the past decades research related to the biodegradation of this drug has allowed the identification and characterization of metabolic intermediates involved in the catabolic pathways of aerobic bacteria (Wu *et al*. 2012).

When paracetamol is used in excess, it can cause liver failure and necrosis due to N-Acetyl-p-benzoquinone imine (NAPQI), a highly toxic paracetamol´s metabolite formed by enzymatic oxidation in the liver (Bessems and Vermeulen, 2001). About 30% and 55% of administered paracetamol is excreted in urine as conjugates paracetamol sulfate and paracetamol glucuronide, respectively (Thomas, 1993). Moreover, a glutathione conjugate (1,4-Michael adduct) of NAPQI, the corresponding cysteine conjugate and mercapturic acid breakdown products have also been found in urine after ingestion of paracetamol (Prescott, 1980). NAPQI is known to be fairly unstable, however, in aqueous solution it readily hydrolyzes into 1,4-benzoquinone, which is another toxic metabolite (Dahlin and Nelson, 1982; Snyder, 2000; Bedner and Maccrehan, 2006). On the other hand, 4-aminophenol (the hydrolytic product of paracetamol) has highly genotoxic and mutagenic effects (Majeska and Holden, 1995; Yoshida *et al*. 1998). The potential toxic effects of paracetamol in aquatic systems, mediated by its reactive oxygen species, have been reported. For example, Antunes *et al*. (2013) studied the effects of paracetamol exposure on physiological traits of bivalves and the results showed a significant increase in all oxidative stress biomarkers, evidencing the onset of deleterious effects.

The detection of paracetamol and its metabolites in surface water suggests that the degree of human use and the disposal in sewage overwhelms their effective removal by conventional sewage treatment (Peake *et al*. 2016). However, there is a lack of information about the environmental behavior of the human metabolites of paracetamol and respective transformation products resulting from their physicochemical and microbial removal in sewage treatment facilities (Peake *et al*. 2016). Yet, given that these metabolites can be persistent, and some are even more toxic than the parent compound, it is essential to determine their fate in biological systems (Wu *et al*. 2012; Marchlewicz *et al*. 2015; Peake *et al*. 2016; Torun *et al*. 2015; Mbokou *et al*. 2016) and, hence, to search for new and effective ways to degrade or remove them from wastewater and resulting treated effluents. In this regard, biodegradation may represent a low-cost effective solution (Wu *et al*. 2012), though several advanced oxidation processes have also been extensively studied for the

degradation of paracetamol (*e.g.* Vogna *et al*. 2002; Andreozzi *et al*. 2003; Fatta-Kassinos *et al*. 2011; Moctezuma *et al*. 2012; Villota *et al*. 2016).

In the beginning of the century little was known about microbial degradation of paracetamol (Pieper and Reineke, 2000). Since then, however, some advances have been made in the knowledge of this subject. When biodegradation is complete (mineralization), the substances are converted to inorganic compounds such as water, carbon dioxide, ammonium, and nitrate, whereas partial breakdown results in the transformation of pharmaceuticals into other metabolites such as the aromatic compounds 4-aminophenol and hydroquinone (and its oxidized form 1,4-benzoquinone) and several carboxylic acids and nitrogen-containing straight chain compounds (Wu *et al*. 2012).

Several microorganisms capable of using paracetamol as a carbon and energy source have been isolated and metabolic pathways for the biodegradation of this drug have been proposed. De Gusseme *et al*. (2011) demonstrated that a membrane bioreactor (MBR) inoculated with an enriched nitrifying bacterial culture was efficient in removing continuously 99.9% paracetamol from a synthetic WWTP effluent spiked with 100 μg/L of this drug. Moreover, in the same work two paracetamol degrading strains identified as *Delftia tsuruhatensis* and *Pseudomonas aeruginosa* were isolated from the MBR biomass and during incubation of these isolates' hydroquinone was formed and considered a potential transformation product. Furthermore, Hu *et al*. (2012) have demonstrated the removal of paracetamol by aerobic granules developed in a sequencing batch reactor. Then Zhang *et al*. (2013) have isolated from these microbial aggregates three bacterial strains of the genera *Stenotrophomonas* and *Pseudomonas* capable of using paracetamol as their sole carbon, nitrogen and energy sources and proposed metabolic pathways for the degradation of this drug, with 4-aminophenol and hydroquinone as first metabolic products. More recently, Karaman *et al*. (2016) demonstrated that paracetamol in activated sludge underwent biodegradation within less than one month and reported that *P. aeruginosa* was the responsible for the biodegradation of paracetamol to 4-aminophenol and hydroquinone. It is worth to note that the identification of bacteria of the genus *Pseudomonas* is common in these various works. In fact, *Pseudomonas* is also known for its ability to degrade other aromatic compounds of environmental concern (Neumann *et al*. 2004; Cámara *et al*. 2009).

Thus, to our knowledge all the microbial catabolic pathways known to be involved in paracetamol biodegradation were studied in a small number of aerobic organisms; therefore, it is possible that unidentified mechanisms with a role in paracetamol biodegradation exist in other organisms. The present study focuses on the characterization of bacterial communities with capacity to biodegrade paracetamol, taking into account the well-known advantages of consortia over pure cultures (Mukred *et al*. 2008), aiming the identification of new genera with a putative role in the

mineralization of this drug. For that purpose bacterial communities obtained from sludge of two types of wastewater treatment plants processes (anaerobic lagoon and oxidation ditch) were tested using media with different nutritional characteristics: multipurpose thioglycolate medium (TGM) generally used for cultivation of anaerobes, and aerobes, mineral salts medium (MSM), modified Postgate B medium specific for sulphate reducing bacteria (SRB) and raw municipal wastewater (MWW), in different oxygenic conditions: anaerobic (sealed flasks), moderate oxygenation (open flasks without aeration) and high oxygenation (open flasks with aeration) according to **Figure 3.1** in order to test the enriched bacterial communities broad from such variety of tested conditions.

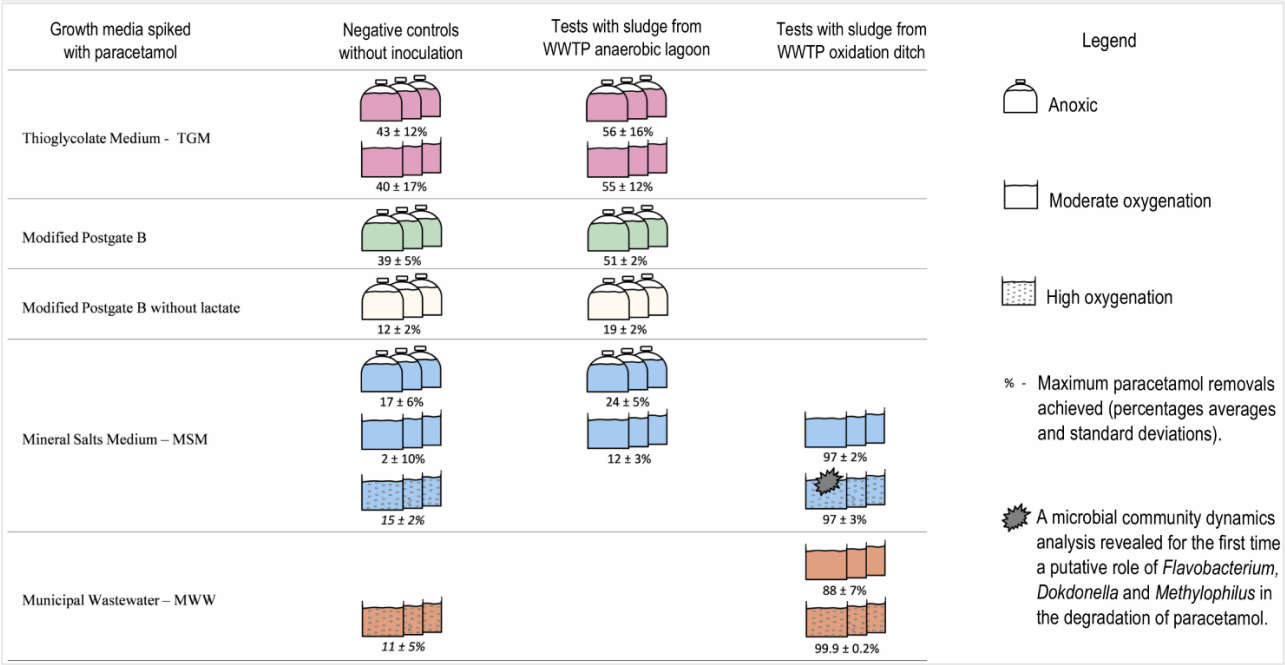

Figure 3.1 Graphical representation of the experiments carried out and features studied in paracetamol biodegradation studies

2. MATERIALS AND METHODS

2.1. Chemicals

The following reagents were purchased from Panreac (Barcelona, Spain): sodium chloride (NaCl), magnesium chloride hexahydrate ($MgCl_2.6H_2O$), calcium chloride ($CaCl_2$), zinc sulfate ($ZnSO_4$), cobalt(II) chloride hexahydrate ($CoCl_2.6H_2O$), manganese(II) sulfate ($MnSO_4$), ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$)), monopotassium phosphate (KH_2PO_4), ammonium chloride (NH_4Cl), sodium sulfate (Na_2SO_4), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), sodium lactate ($C_3H_5NaO_3$), ammonium hydroxide (NH_4OH). Ethylenediamine tetraacetic acid (EDTA), potassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) both AnalaR NORMAPUR® were obtained from VWR Prolabo Chemicals (Leuven, Belgium). Yeast extract was acquired from HiMedia Laboratories (Mumbai, India) and thioglycolic acid was

purchased from Merck (Darmstadt, Germany). All the solutions of paracetamol (99% purity), hydroquinone (99% purity), 4-aminophenol (97% purity), 4-nitrophenol (99% purity) were purchased from Sigma-Aldrich (Deisenhofer, Germany). Acetonitrile (ACN) and methanol, both HPLC grade, and phosphoric acid (85% purity) were supplied by VWR Prolabo Chemicals (Fontenay-sous-Bois, France) and formic acid (HCOOH) high purity grade was obtained from Amresco (Solon, USA). Phosphate buffer solution of pH = 4.88 was prepared by dissolving 4.5 g KH_2PO_4 and 0.0412 g $K_2HPO_4.3H_2O$ in 500 mL of ultra pure water, using phosphoric acid (85%) to adjust pH if necessary.

2.2. Inocula source and preparation

Sludges from two Portuguese WWTPs with different processes were used as inocula sources. Sludge collected in the Faro East WWTP's lagoon system without aeration (anaerobic sludge) was used for a first set of experiments under anaerobic and moderate aerobic conditions, while sludge from the Faro Northwest WWTP's oxidation ditch with aeration (aerobic sludge) was used in a second set of experiments in which highly aerobic conditions were tested (**Figure 3.1**).

To enrich the inoculum from the lagoon system, 2 g of sludge sediment were inoculated on each of two separate 250 mL culture flasks containing 200 mL of TGM medium. In one flask 10 mL of paraffin-oil was added to prevent gas diffusion and the flask was sealed with a butyl rubber stopper and an aluminum crimp seal to avoid air intake in order to create anaerobic condition. In the other flask, cotton was used to serve as lid, thus allowing the maintenance of aerobic conditions. To enrich the inoculum from the oxidation ditch, 2 mL of water with sludge was inoculated in just one flask with 200 mL TGM medium with cotton as lid. The anaerobic flask was kept without shaking and the two aerobic flasks were placed in an orbital shaker at a speed of 150 rpm; all were maintained at room temperature and grown for 24 hours. The anaerobic enriched culture was used to inoculate all tests under anaerobic conditions. The aerobic enriched cultures from each type of sludge were used to inoculate the respective tests under moderate and highly aerobic conditions.

In the inocula enrichments, initial and final optical densities were measured at 600 nm (OD_{600nm}) as absorbance using a Hach-Lange spectrophotometer DR-2800 (Sköndal, Sweden). The initial OD_{600nm} values of enrichment media immediately after inoculation with the anaerobic lagoon and the oxidation ditch sludges were similar: 0.286 and 0.264, respectively. Thus, the also similar OD_{600nm} values measured after the 24 hours of incubation at room temperature in all enrichment conditions (anaerobic lagoon sludge/anaerobic growth = 0.838; anaerobic lagoon sludge/aerobic growth = 0.831; oxidation ditch sludge/aerobic growth = 0.833) roughly indicates similar bacterial growths.

A SRB consortium enriched under anaerobic conditions for 7 days in Postgate B medium from the sludge sample collected in the anaerobic lagoon system of Faro East WWTP was used to test the paracetamol biodegradation ability of that particular anaerobic community. The initial and the final OD_{600nm} values in the SRB inoculum enrichment were 0.165 and 0.657, respectively.

Previous steps of sludge washing were carried out to eliminate dissolved carbon compounds that could serve as carbon and energy sources for bacterial growth because in some experiments (with MSM and with the modified Postgate B without lactate) paracetamol was expected to be the only carbon source. With that purpose, the enriched inocula coming from the anaerobic and aerobic sludges were centrifuged at 2500 × g for 10 min at room temperature, the supernatant was discarded, and the pellets re-suspended using the MSM. This procedure was repeated twice before inoculation of test cultures.

2.3. IC_{50} of paracetamol for bacterial growth

The half maximal inhibitory concentration (IC_{50}) for bacterial growth, the concentration of paracetamol that causes a 50% drop in bacteria growth, was estimated from duplicate batch cultures in TGM under aerobic and under anaerobic conditions with different paracetamol concentrations (4, 6, 8, 10 and 12 g/L) and without paracetamol (as a reference for normal bacterial growth) during an incubation period of 24 h at room temperature. Aerobic sludge was used as an inoculum for the aerobic cultures and anaerobic sludge for the anaerobic cultures.

The bacterial growth for IC_{50} determination was analyzed by measuring the OD_{600nm} using a Hach-Lange spectrophotometer DR-2800 (Sköndal, Sweden). Based on the OD_{600nm} values, the percentage of bacterial viability (OD_{600nm} in a culture with a drug concentration over the OD_{600nm} in the culture without drug) was calculated for the different tested paracetamol concentrations. IC_{50} values were estimated using GraphPad Prism 6 Software published by GraphPad Software, Inc. using dose response curve log (inhibitor) *vs*. normalized response-variable slope analyses. For both anaerobic and aerobic assays, bacterial viability percentage (%) *versus* logarithm of paracetamol concentration (g/L) were plotted and best fitting sigmoidal curves were used to calculate IC_{50} values.

2.4. Biodegradation of paracetamol

Anaerobic and aerobic cultures without aeration as well as aerated cultures were evaluated in order to mimic the conditions in WWTPs lagoon systems and in WWTPs with oxidation ditch.

The cultures were carried out in triplicated batches with 10% (v/v) sludge enriched inoculum on five different media spiked with paracetamol: (i) TGM, an universal complex medium; (ii) MSM, a medium without carbon compounds composed of 1 g/L K_2HPO_4, 1 g/L KH_2PO_4, 20 mg/L NaCl, 0.41 g/L $MgCl_2.6H_2O$, 0.04 g/L $CaCl_2$, 1.53 mg/L $ZnSO_4$, 0.78 g/L $CoCl_2.6H_2O$, 0.74 mg/L

$MnSO_4$, 20 mg/L EDTA and 0.64 mg/L $(NH_4)_6Mo_7O_{24}.4H_2O$); (iii) a modified Postgate B medium used for SRB composed of 0.5 g/L KH_2PO_4, 1 g/L NH_4Cl, 1 g/L Na_2SO_4, 1 g/L yeast extract, 0.1 g/L ascorbic acid, 0.1 g/L thioglycolic acid, 2 g/L $MgSO_4.7H_2O$ and 7.75 g/L of the carbon source $C_3H_5O_3Na$ – sodium lactate; (iv) the modified Postgate B medium without the carbon source; and (v) raw MWW, a sample collected at the entry of the Faro Northwest WWTP.

All media, except the raw MWW, were autoclaved (120 ºC for 45 min) and cooled to room temperature before the addition of paracetamol and inoculation. Paracetamol was added using a PES syringe filter of 0.2 µm pore size from VWR (Leuven, Belgium) to make the tested drug concentrations: between 10 mg/L and 100 mg/L, which are in the range of values reported in European WWTP influents (*e.g.* Roberts *et al*. 2006; Papageorgiou *et al*. 2016; Vymazal *et al*. 2017), as well as a much higher concentration (10000 mg/L), which is above the IC_{50} values estimated in this work for bacterial growth.

Negative controls (without sludge inoculum) were performed with all tested media to evaluate the eventual degradation of these compounds in ways other than the biological route, or to check eventual interactions of the drug with the medium. Moreover, to evaluate the possible adsorption of paracetamol on the sludge used as inoculum, independent assays were performed with autoclaved (120 ºC for 45 min) sludge in MSM and MWW media with 50 mg/L of this drug.

All the assays were carried out in triplicated in the dark (to avoid photodegradation) at room temperature and at atmospheric pressure. To create anaerobic conditions, liquid paraffin was added to cultures and the batch flasks were sealed with butyl rubber stoppers and aluminum crimp seals. The liquid paraffin prevents gas diffusion through the medium surface and the flask sealed with a butyl rubber stopper and an aluminum crimp seal avoids air intake into the flask; thus, the oxygen initially present is rapidly consumed and anaerobic conditions are created. In the anaerobic tests resazurin was added as an indicator to confirm the absence of oxygen. To maintain aerobic conditions with moderate oxygenation, the cultures were incubated in open batch flasks without stirring or shaking. To achieve aerobic conditions with high oxygenation levels, cultures were carried out in open batches with aeration (110 mL/min airflow).

The averages and standard deviations of dissolved oxygen (DO) percent saturation in the various tests under aerobic conditions were calculated using measurements made in the respective replicate cultures with a portable CD650 meter (Eutech Instruments) after 24 hours of incubation since inoculation. For moderate oxygenation conditions in open flasks without aeration, the DO percent saturation was: 9.3 ± 1.8% in the tests with TGM, 31.1 ± 2.3% in the tests with MSM and 27.5 ± 2.2% in the tests with MWW (24 hours after both first media were inoculated with the anaerobic lagoon sludge and the third medium inoculated with the oxidation ditch sludge). For high

oxygenation conditions in aerated flaks, the DO percent saturation was: 94.8 ± 1.6% in the tests with MSM and 95.5 ± 1.3% in the tests with MWW (24 hours after both these media were inoculated with the oxidation ditch sludge).

The experiments in TGM, MSM and MWW were incubated during 72 h. The experiments in modified Postgate B were maintained for 14 days, the time to assure high SRB activity and total reduction of the sulphate in the growth media, according to the experience of the research group with these bacteria, namely for acid mine drainage bioremediation purposes (Costa and Duarte, 2005; Da Costa *et al*. 2013; Vitor *et al*. 2015). Aiming to evaluate the SRB activity, the cultures in modified Postgate B and in modified Postgate B without lactate were monitored for pH and redox potential (E_h) using a GLP 21 pH meter, Crison (Barcelona, Spain) and for the sulphate concentration through molecular UV/Visible spectroscopy at 450 nm using a Hach-Lange™ DR 2800 spectrophotometer (Sköndal, Sweden) and the sulfaVer4 method from Hach-Lange (Düsseldorf, Germany).

One-Way ANOVA (Single Factor) tests using Excel Data Analysis Tools were performed to evaluate if differences between the inoculated test cultures and the respective non inoculated negative controls were significant for 5% error ($\alpha = 0.05$).

2.5. Effect of paracetamol on chemical oxygen demand (COD) degradation

COD is often used to measure organic matter, allowing an indirect quantification of the amount of oxidizable compounds in wastewaters, treated effluents and receiving waters.

The COD (mg O_2/L) was analyzed in assays with MWW medium in the presence and absence of paracetamol to evaluate the influence of this drug on the degradation of the organic matter present in wastewater. COD was also determined in the MSM medium assays with paracetamol (i) inoculated with aerobic sludge and (ii) without inoculum, to evaluate the variation of COD caused by adding this drug and by its removal. Samples collected immediately after preparation of these assays and after 72 h incubation were used for COD analysis.

Measurements were carried out using cuvette tests for the dichromate method with LCK 514 kit, purchased from Hach-Lange (Düsseldorf, Germany). Tubes with pre-determined amounts of potassium dichromate, sulphuric acid and silver sulphate as catalyst were homogenized and mixed with 2 mL of samples. The samples were digested in an AccuBlock™ Digital Dry Bath, Labnet (Massachusetts, USA) at 148 ºC during 120 min. Samples were allowed to cool to room temperature and COD values were measured at 605 nm using a Hach-Lange™ DR 2800 spectrophotometer (Sköndal, Sweden).

2.6. HPLC analysis

Immediately after collecting the samples, they were filtered with 0.2 µm polypropylene (PP) syringe filters from VWR (Leuven, Belgium) and then stored overnight at 4 ºC before chromatographic analysis.

Paracetamol and respective metabolic products were analyzed using a modular Advanced Scientific Instrument KNAUER HPLC system with a Smartline UV detector 2600 Smartline Manager 5000 (Berlin, Germany). The output signal was monitored and integrated using ClarityChrom® software. The compounds were separated using a reversed phase Xbridge-C18 column (250 × 4.6 mm, 5 µm particle size) - Hybrid technology connected to a Guard Column Xbridge-C18 column (4.6 × 200 mm, 5 µm particle size), both purchased from Waters Corporation (Milford, MA, USA).

2.6.1. Quantification of paracetamol

To analyse the biodegradation of paracetamol, HPLC analysis was performed with a fast isocratic method using a mobile phase consisted of ACN:water (25:75, v/v) adjusted to pH 3.74 with orthophosphoric acid (85%) using a flow rate of 1.0 mL/min and a run time of 5 minutes with the column maintained at room temperature. The injection volume was 20 µL and a wavelength of 244 nm was used for detection.

Exclusive standard calibration curves were constructed for each experiment to determine the concentrations of paracetamol in the corresponding samples. The concentration ranges of paracetamol standards, prepared with the respective medium were: TGM medium: 5 to 100 mg/L and 50 to 2500 mg/L, depending on the paracetamol concentration tested; MSM medium: 5 to 1000 mg/L; Modified Postgate B medium: 1 to 110 mg/L; MWW assays: 5 to 250 mg/L. The limits of detection (LOD) were determined by the analysis of standards with known concentrations to establish the minimum level at which the analyte peak could be reliably detected by visual evaluation, as described in the harmonized tripartite guideline (ICH, 1996). LODs of 1, 0.5, 0.15 and 0.2 mg/L were estimated for TGM, MSM, modified Postgate B and MWW, respectively.

2.6.2. Identification of paracetamol metabolic products

Samples from the experiments in which high degradation of paracetamol was observed when using the fast isocratic HPLC method were run with a longer method that allows a good separation, and therefore identification, of products known to be generated from the degradation of this drug. This analysis was performed with the mobile phase potassium-phosphate-buffer (pH 4.88):methanol, which was optimized by Calinescu *et al*. (2012), and the following gradient program with respective mobile phase ratios (v/v): 1st ramp from 0 to 8 min with 80:20 to 50:50 (v/v); 1st stationary step from 8 to11 min with 50:50 (v/v); 2nd ramp from 11 to 12 min with 50:50 to 80:20

(v/v); 2nd stationary step from 12 to 15 min with 80:20 (v/v). The injection volume was 20 µL, the flow rate was set at 0.8 mL/min, the column was maintained at room temperature and detection was performed at 244 nm.

2.7. Characterization of paracetamol degrading bacterial communities

Microbial communities were studied along the experiment in one culture of the three replicates with MSM, in which paracetamol (50 mg/L) was the only carbon compound added to the medium, aiming to identify microorganisms with a putative role in the degradation of this drug. As the three replicate cultures were inoculated exactly with the same microbial community (source: enriched culture from WWTP's oxidation ditch sludge; quantity: 10% (v/v)) and were maintained under exactly the same conditions (aeration: a shared air pump; temperature and light: flasks maintained side by side), it was assumed that studying the microbial dynamics through massive sequencing of 16S rRNA genes on just one of the cultures would provide enough robust evidences to identify taxa putatively involved in the degradation of paracetamol and/or its metabolites.

For that purpose, DNA was extracted from culture samples with the PowerSoil® DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), which uses bead beating and silica spin filter technology for extraction. The concentration and quality of eluted DNA was determined using a NanoDrop spectrophotometer Thermo Scientific 3300 and DNA samples were sent to the company DNASense ApS (Aalborg, Denmark) for large-scale biodiversity analysis through massive parallel sequencing of 16S rRNA genes according to the following procedures.

2.7.1. 16S rRNA amplicon library preparation

The procedure for bacterial 16S ribosomal ribonucleic acid (rRNA) amplicon sequencing targeting the V1-3 variable regions is based on Caporaso *et al*. (2012) using primers adapted from the Human Gut Consortium (Ward *et al*. 2012). Ten nanograms of extracted DNA was used as template and the polymerase chain reaction (PCR) (25 µL) contained deoxynucleotide (dNTPs) (400 nM of each), $MgSO_4$ (1.5 mM), Platinum® Taq DNA polymerase HF (2 mU), 1X Platinum® High Fidelity buffer (Thermo Fisher Scientific, USA), and barcoded library adaptors (400 nM) containing V1-3 specific primers: 27F AGAGTTTGATCCTGGCTCAG and 534R ATTACCGCGGCTGCTGG. PCR settings: Initial denaturation at 95 °C for 2 min, 30 cycles of 95 °C for 20 s, 56 °C for 30 s, 72 °C for 60 s and final elongation at 72 °C for 5 min.

All PCR reactions were run in duplicate and pooled. The amplicon libraries were purified using the Agencourt® AMpure XP bead protocol (Beckmann Coulter, USA) with the following exceptions: the sample/bead solution ratio was 5/4, and the purified DNA was eluted in 33 µL nuclease-free

water. Library concentration was measured with Quant-iT™ HS DNA Assay (Thermo Fisher Scientific, USA) and quality validated with a Tapestation 2200, using D1K ScreenTapes (Agilent, USA). Based on library concentrations and calculated amplicon sizes, the samples were pooled in equimolar concentrations and diluted to 4 nM.

2.7.2. DNA sequencing

The samples were paired end sequenced (2 x 301bp) on a MiSeq (Illumina) using a MiSeq Reagent kit v3, 600 cycles (Illumina) following the standard guidelines for preparing and loading samples on the MiSeq. 10% Phix control library was spiked in to overcome low complexity issue often observed with amplicon samples.

2.7.3. 16S rRNA amplicon bioinformatic processing (bacteria V1-3)

Forward and reverse reads were trimmed for quality using Trimmomatic v. 0.32 (Bolger *et al*. 2014) with the settings SLIDINGWINDOW:5:3 and MINLEN:275. The trimmed forward and reverse reads were merged using FLASH v. 1.2.7 (Magoc and Salzberg, 2011) with the settings -m 25 -M 200. The merged reads were dereplicated and formatted for use in the UPARSE workflow (Edgar, 2013). The dereplicated reads were clustered, using the usearch v. 7.0.1090 -cluster_otus command with default settings. Operational taxonomic unit (OTU) abundances were estimated using the usearch v. 7.0.1090usearch_global command with -id 0.97. Taxonomy was assigned using the RDP classifier (Wang *et al*. 2007) as implemented in the parallel_assign_taxonomy_rdp.py script in QIIME (Caporaso *et al*. 2010), using the MiDAS database v.1.20 (McIlroy *et al*. 2015). The results were analyzed in R (R Core Team, 2015) through the Rstudio IDE using the ampvis package v.1.24.0 (Albertsen *et al*. 2015) (**Table S1**; **Annex 1**).

The raw massive sequencing data from 16S rRNA gene amplicons generated in this work was archived in the NCBI Sequence Read Archive (SRA) database under SRA study SRP125056 and BioProject PRJNA418409. The OTU sequences were published with GenBank accession numbers MG554745 to MG557554 (**Table S2**; **Annex1**).

3. RESULTS AND DISCUSSION

3.1. IC_{50} of paracetamol for bacterial growth

The studies started by the determination of paracetamol IC_{50} for bacterial growth, which is the concentration of this drug causing a drop of 50% in bacterial viability (defined as the percentage of the OD_{600nm} measured in a culture with a certain paracetamol concentration in relation to the OD_{600nm} in the control culture without the drug). Estimates were based on measurements in cultures

with the general growth medium TGM containing different concentrations of paracetamol, under anaerobic and aerobic conditions.

The bacterial viability as a function of the logarithm of paracetamol concentration fits reasonably for both conditions a sigmoidal curve (**Figure 3.2**).

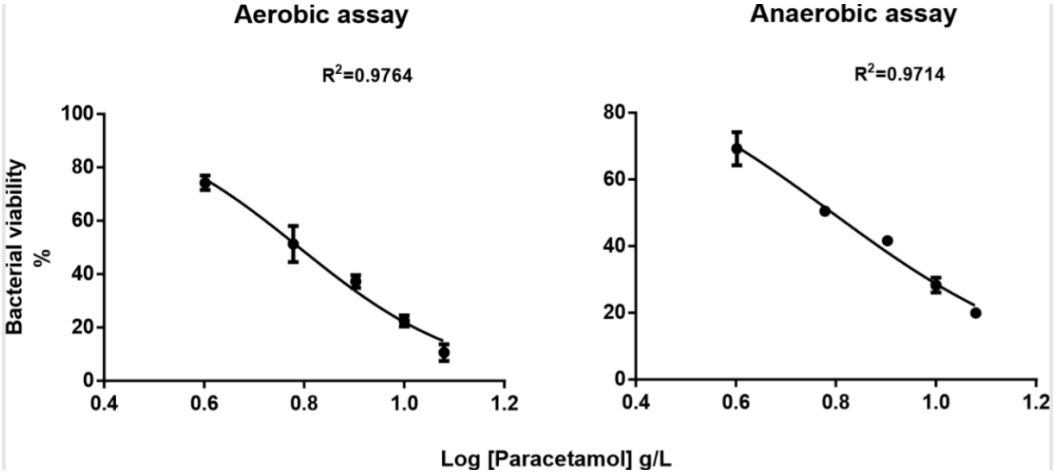

Figure 3.2 Graphical representation of best fitting sigmoidal curves for bacterial viability (%) as a function of logarithm of paracetamol concentration (g/L), for aerobic and anaerobic assays, with values expressed as mean ± distance to individual data points (n=2). The percentage of bacterial viability is the optical density at 600 nm (OD_{600nm}) measured in a culture with a certain drug concentration divided by the OD_{600nm} in the control culture without drug, multiplied by 100. In some points the bars are smaller than the symbol, therefore not visible

Those functions were similar; therefore, the IC_{50} values for both conditions are also similar: 6.20 and 6.162 g/L, respectively for anaerobic and aerobic conditions. These IC_{50} values are much higher than the concentrations usually reported in raw untreated wastewaters of less than 0.2 g/L (*e.g.* Roberts *et al*. 2006; Papageorgiou *et al*. 2016; Vymazal *et al*. 2017). Thus, problems in WWTPs caused by paracetamol affecting the bacterial communities that degrade the organic matter are not expected and have not yet, to our knowledge, been reported.

3.2. Biodegradation of paracetamol

The results obtained clearly indicate that the major routes for the biodegradation of this drug in WWTPs involve aerobic organisms; some already reported previously and others identified in this work for the first time, as discussed further below. However, some slow biodegradation seems to occur under anaerobic conditions, which was not more evident due to the short time of the assays.

The overall results obtained in the tests performed to evaluate paracetamol biodegradation under diverse experimental conditions are summarized in **Table 3.1**.

Table 3.1 Percentages of paracetamol removal in each assay after 24 and 72 hours of incubation (7 and 14 days, respectively, for tests with modified Postgate B medium). Values are expressed as the mean ± standard deviation (n=3)

Conditions			24 hours incubation [a]			72 hours incubation [b]		
Growth media	Oxygenic	Paracetamol concentrations (mg/L)	No sludge *or* *autoclaved sludge* [c] (negative control)	Sludge from Faro East WWTP anaerobic lagoon	Sludge from Faro Northwest WWTP oxidation ditch	No sludge *or* *autoclaved sludge* [c] (negative control)	Sludge from Faro East WWTP anaerobic lagoon	Sludge from Faro Northwest WWTP oxidation ditch
TGM	Anaerobic	10	17 ± 3%	24 ± 9%	NT	43 ± 12%	56 ± 16%	NT
		50	21 ± 5%	22 ±13%		37 ± 10%	45 ±20%	
		80	2 ± 5%	12 ± 8%		25 ± 9%	33 ± 9%	
		10000	13 ± 4%	22 ± 14%		11 ± 10%	26 ± 12%	
	Aerobic without aeration	10	40 ± 17%	55 ± 12%	NT	31 ± 8%	45 ± 8%	NT
		50	22 ± 11%	25 ± 2%		25 ± 14%	44 ± 5%	
		80	27 ± 6%	33 ± 7%		8 ± 13%	16 ± 5%	
		10000	22 ± 11%	25 ± 3%		20 ± 11%	22 ± 1%	
Modified Postgate B	Anaerobic	10	5 ± 3%	8 ± 2%	NT	7 ± 2%	18 ± 2%*	NT
		80	32 ± 10 %	44 ± 2%		39 ± 5 %	51 ± 2%*	
		100	17 ± 3%	31 ± 4%*		23 ± 2%	33 ± 6%*	
Modified Postgate B without lactate	Anaerobic	100	7 ± 3%	10 ± 4%	NT	12 ± 2%	19 ± 2%*	NT
MSM	Anaerobic	10	0.8 ± 8%	6 ± 10%	NT	NR	6 ± 10%	NT
		50	NR	NR		14 ± 6%	24 ± 5%	
		80	NR	8 ± 13%		17 ± 6%	21 ± 4%	
	Aerobic without aeration	10	2 ± 10%	10 ± 9%	NT	2 ± 10%	10 ± 9%	NT
		50	NR	3 ± 4%	NR	3 ± 3%	12 ± 3%*	97 ± 2%*
		80	NR	NR	NT	0.4 ± 11%	9 ± 5%	NT
	Aerobic with aeration	50	1 ± 1% NR [c]	NT	3 ± 3%	3 ± 8% 15 ± 2% [c]	NT	97 ± 3%*
MWW	Aerobic without aeration	50	NT	NT	11 ± 10%	NT	NT	88 ± 7%
	Aerobic with aeration	50	6 ± 2% [c]	NT	70 ± 10%*	11 ± 5% [c]	NT	99.9 ± 0.2%*

a, b = 7 and 14 days, respectively, for tests with modified Postgate B medium; c = negative controls carried out with autoclaved sludge.
* = paracetamol removal is significantly different (one-way ANOVA for 5% error) from the observed in the respective negative control.
NR = no removal; NT = not tested.

3.2.1 TGM Medium

The TGM medium is a complex and non-selective medium for cultivation of anaerobes, aerobes and microaerophiles, thus it allows the growth of a wide diversity of bacteria. The main electron acceptor in TGM is oxygen in the tests under aerobic conditions, while oxidized forms of

inorganics such as nitrate, sulphate, iron (III) and manganese (IV), as well as amino acids and glucose are the potential electron acceptors in the tests under anaerobic conditions. This medium was chosen to test one paracetamol concentration (10000 mg/L) above the IC_{50} values of this drug estimated in this work for bacterial growth (6204 and 6162 mg/L) and concentrations of 10, 50 and 80 mg/L. Anaerobic and moderate aerobic (open flasks without aeration) were tested with TGM using the inoculum enriched from the WWTP lagoon system sludge.

In the inoculated TGM cultures under anaerobic conditions with the lower tested paracetamol concentrations of 10, 50 and 80 mg/L, drug removals of 24%, 22% and 12% (respectively) were observed after 24 h and removals around twice these values (56%, 45% and 33%) were achieved after 72 h (**Table 3.1**). With the same conditions but in the presence of 10000 mg/L paracetamol, a removal efficiency of 22 ± 14% was attained after 24 h assay, but no major changes were observed after 72 h (26 ± 12%). According to the plotted IC_{50} curves (**Figure 3.2**) the bacterial viability (%) for 10000 mg/L paracetamol is low (20% to 30%). Therefore, probably this high concentration inhibited the growth of bacteria degrading this drug. Even so, in all these cases and for both sampling days, the differences in paracetamol removal averages between the inoculated and non-inoculated tests were relatively low (< 15%) and not significant (for 5% error), suggesting that biodegradation had a limited role in the removal of this drug.

In the inoculated TGM medium under aerobic conditions with moderate oxygenation (open flasks without aeration), the differences in paracetamol removal between the inoculated and non-inoculated tests were also low (< 20%) and not significant (for 5% error), thus suggesting a limited role of biological activity in the drug removal also in this case.

In summary, in the experiments with TGM media, three days were necessary to achieve paracetamol removals around 55% under anaerobic conditions while in moderate aerobic conditions this level of removal was achieved after only one day, and in both these conditions a small difference was observed between the inoculated tests and the non-inoculated negative controls. Paracetamol resistance to biodegradation and biotransformation under anaerobic conditions was already described (*e.g.* Musson *et al*. 2010). Moreover, these authors also reported that measurable losses from solution may occur through abiotic mechanisms as described in BioWin, an anaerobic degradation prediction model developed by the US Environmental Protection Agency (Musson *et al*. 2010).

3.2.2. Modified Postgate B

This medium is optimized to cultivate SRB under anaerobic conditions, thus the main electron acceptor in its composition is sulphate. For all tested paracetamol concentrations (10, 80 and 100 mg/L) in cultures using modified Postgate B medium the pH remained within the interval of 6.5 to

7.5, which is in the optimum range for SRB. Indeed, the parameters analyzed to monitor these bacteria indicated high activity in the inoculated tests: the redox potential (E_h) evolved to values between -350 and -400 mV after 2 weeks incubation under anaerobic conditions and the sulphate removal in this period was equal or higher than the 72% observed in the control culture without the drug. These results demonstrate that the inoculated SRB consortium enriched from the Faro East WWTP's lagoon system sludge was resistant to all concentrations of paracetamol tested. In what concerns the degradation of paracetamol, the results were slightly different from those observed in the tests with TGM. The maximum removal of paracetamol achieved was in the same range (51 ± 2%) and the differences on the removal averages of this drug between the inoculated cultures and the respective non inoculated negative controls were also small (less than 15%). However, despite small, those differences were significant (for 5% error) after 14 days incubation for all paracetamol concentrations tested.

In the assay performed using the same SRB inoculum in modified Postgate B medium without lactate and spiked with 100 mg/L of paracetamol, the pH also remained neutral (7.0 ± 0.2) in all cultures and the redox potential (E_h) in the inoculated tests also evolved to values between -350 and -400 mV, which are optimal conditions for SRB. However, the concentration of sulphate only decreased 25%, indicating low SRB activity. In any case, the values of paracetamol removal achieved were still low (19 ± 2%) and only slightly higher than in the non-inoculated negative controls (12 ± 2%). Nevertheless, this small difference achieved after 14 days of incubation was significant (for 5% error).

These to some extent higher removals of paracetamol observed in the tests inoculated with the SRB enriched consortium compared to the respective non inoculated negative controls may indicate the existence of mechanisms associated with biological activity contributing to paracetamol removal. However, it remains unknown whether paracetamol was degraded via metabolic pathways, or if it reacted with compounds released by the active microbial communities. Despite this, it seems that the SRB present in this consortium are not directly involved in the removal because it occurred at the same extent both in medium with lactate (with high SRB activity) and without lactate (with low SRB activity). Thus, probably other microorganisms than SRB, eventually less abundant in the inoculum, may have been directly or indirectly responsible for the observed paracetamol removal.

3.2.3. MSM medium

This medium, exclusively composed by inorganic compounds, was used to test paracetamol biodegradation when this drug was the only carbon source available. The putative main electron acceptors of MSM are oxygen, in the tests under aerobic conditions, and nitrate sulphate and

manganese (IV), in the tests under anaerobic conditions. Experiments with MSM were first performed using sludge from the WWTP lagoon system as inoculum for the anaerobic and moderate aerobic conditions. In these tests, minor (< 25%) or no degradation of paracetamol was observed either after 24 or 72 h of incubation with the three concentrations of paracetamol tested (10, 50, and 80 mg/L). Moreover, in these conditions the non-significant (for 5% error) and small differences (<10%) in paracetamol removal obtained between the negative controls and the inoculated cultures, reinforce the idea that there was no degradation, or just small degradation, due to biological activity, as observed in the experiments with TGM.

The partial removals of paracetamol achieved when the WWTP lagoon sludge was used as inoculum in TGM and MSM under anaerobic and moderate aerobic conditions and the negligible contribution of the inoculum in these removals suggest that the conditions of growth and/or the nature of the inoculum probably hampered the biodegradation of the total amount of the drug in the tested cultures. Therefore, trying to improve paracetamol biodegradation, further experiments were carried out in highly oxygenated (aerated) aerobic conditions and using as inoculum sludge from a WWTP's oxidation ditch. One concentration of paracetamol (50 mg/L) was tested in aerated MSM inoculated with this sludge (**Figure 3.3**).

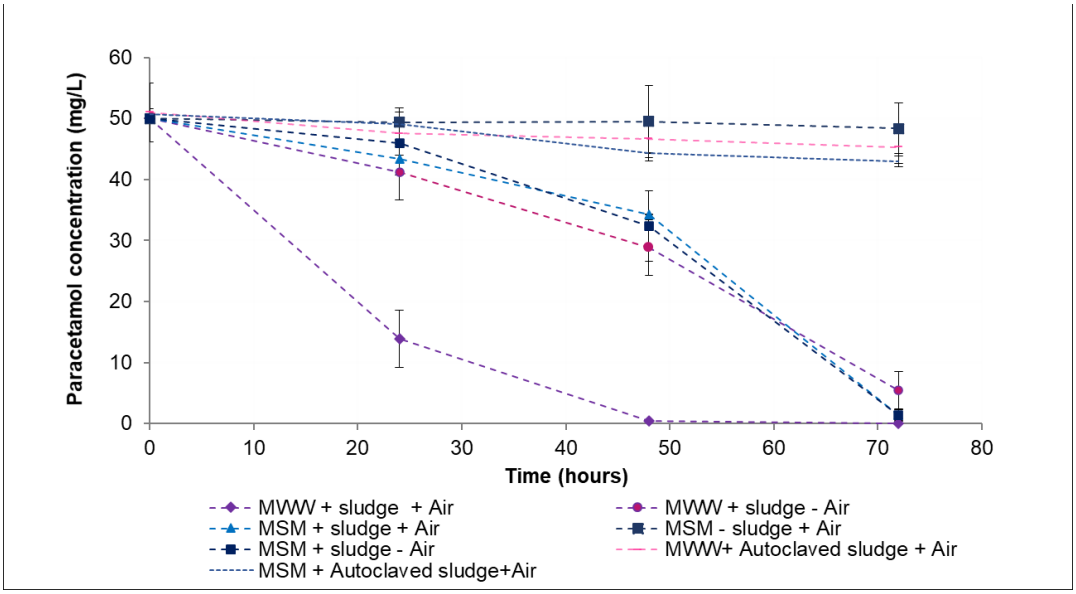

Figure 3.3 Profiles of paracetamol concentration as a function of time in the biodegradation experiments with MSM and MWW using sludge from the WWTP oxidation ditch as inoculum. Inoculated cultures (+ sludge), negative control without inoculum (– sludge), aerated cultures (+ air) and cultures in open flasks but without aeration (– air). Values are expressed as the mean ± standard deviation (n=3). In some points the bars are smaller than the symbol, therefore not visible

After 24 h of incubation only 3 ± 3% of the drug was removed, which was not significantly different (for 5% error) from the 1 ± 1% removal observed in the negative control. However, after 72 h of incubation a removal of 97 ± 2% was achieved, while practically no removal (3 ± 8%) was

still observed in the non-inoculated negative controls, indicating that the major cause of paracetamol removal was due to inoculation with sludge. An additional test with autoclaved sludge in MSM was performed to evaluate the possible adsorption of paracetamol to sludge. The results revealed just a small drug removal (15 ± 2%), reinforcing the idea that the major cause of paracetamol removal using MSM medium in highly oxygenated conditions was the biological activity of aerobic microorganisms (**Figure 3.3**).

3.2.4. MWW Media

MWW was used in order to mimic the real conditions in WWTPs. It was tested just under aerobic conditions, in which the main electron accepter is oxygen. Cultures with 50 mg/L paracetamol were tested under moderate aerobic conditions and highly oxygenated aerobic conditions (as described above) using sludge from the WWTP oxidation ditch as inoculum. After 24 h incubation just 11 ± 10% paracetamol was removed from MWW without aeration and 70 ± 10% was removed with aeration, whereas after 72 h the removal was nearly complete in both cases: 88 ± 7% and 99.9 ± 0.2%, respectively **(Table 3.1**). In this experiment, negative controls without sludge were not carried out because the MWW is itself a source of microbial inoculum, but a test with both autoclaved MWW and sludge was performed. The low paracetamol removal observed in that test (6 ± 2% and 11 ± 5% for 24 h and 72 h incubation, respectively) is significantly different from that achieved in the tests with active sludge, which indicates that biological degradation was the main mechanism involved in paracetamol removal using MWW medium under highly oxygenated conditions, as happened in the tests with highly oxygenated MSM described above.

3.2.5. General analysis of biodegradation experiments

The results suggest that paracetamol removal in the assays under anaerobic and moderate aerobic conditions using sludge from the Faro East WWTP lagoon system was mainly due to other causes than microbial activity: removal was always below 60% and was, in general, similar to, or weakly higher than, the obtained in the respective negative controls (without microbial inoculum). Moreover, the results of the non-inoculated tests show higher removals for TGM (removals up to 43 ± 12%) and modified Postgate B medium (removals up to 39 ± 5%) than for the simpler MSM medium (removals below 17 ± 6%), suggesting that some components in the most complex media were able to chemically react with paracetamol, contributing for its removal. One non biological mechanism that can account for the observed losses of paracetamol in all tested media is its oxidation by SO_4^{2-} ions. It is known that compared with hydroxyl radical ($^{\bullet}OH$), SO_4^{2-} is more selective for oxidation by electron-transfer reaction and more powerful for the decomposition of contaminants at neutral pH (Mezyk *et al*. 2011; Zhang *et al*. 2015). Indeed, when

peroxymonosulfate (PMS) is activated by catalysts it decomposes generating SO_4^{2-}, which degrades pollutants. Thus, several catalysts have been explored for PMS oxidation and some attracted great interest due to its remarkable separation and catalysis, such as spinel ferrites of MFe_2O_4 (M = Fe, Mn, Co, Ni, and Cu), (Tan *et al*. 2017). Another mechanism putatively accounting for the decrease of paracetamol detection in the experiments with TGM (including in the non-inoculated controls) is the possible formation of protein-paracetamol complexes. It has been shown that paracetamol and serum proteins form complexes (*e.g.* Daneshgar *et al*. 2009) and the TGM has beef extracts in its composition.

On other hand, the results from the assays under moderate aerobic and highly aerobic conditions inoculated with sludge from the Faro North WWTP oxidation ditch indicate that in this case aerobic microbial activity played a major role in the removal of paracetamol: (i) the final removal of paracetamol was always higher than 85% in the inoculated tests while in the respective negative controls (without sludge inoculum or with autoclaved sludge) it was small (< 20%) and (ii) within tests with the same media (MSM or MWW) the removal of paracetamol was faster under highly aerobic conditions (aerated cultures) than in the moderate aerobic conditions (non-aerated open cultures). Thus, in these assays' paracetamol concentrations were also studied in samples collected after 48 h of incubation for a better analysis of its degradation over time. With that it was possible to verify that in the inoculated MWW under aeration, paracetamol removal was already complete (99.1 ± 0.4%) after 48 h, which did not happen in the non-aerated MWW cultures, neither in both the aerated and non-aerated MSM cultures (**Figure 3.3**). That may be attributed to three causes: (i) MWW was itself an additional source of microorganisms involved in the biodegradation of paracetamol; (ii) MWW contained compounds, not present in MSM, which play a role in biological pathways contributing to the biodegradation of this drug and (iii) MWW contained compounds, also not present in MSM, which participate in chemical reactions contributing to a non-biological removal of the drug.

Though the biomass growth was not analyzed during the biodegradation tests, it was evaluated by optical density measurements at the beginning and at the end of the inocula enrichment in TGM (a rich medium for anaerobic and aerobic microorganisms) and during that period roughly similar growths were observed in the different redox conditions (as described in **section 2.2 of Material and Methods**). Taking that into account, together with the fact that roughly similar biomasses were inoculated in all tests, it can be assumed that at least for the tests with TGM the bacterial growth under anaerobic conditions was not a limiting factor. However, when MWW was used, the lower paracetamol degradation rates observed under low oxygenic conditions (27.5 ± 2.2% DO) compared with those observed under high oxygenic conditions (95.0 ± 1.3% DO) were probably the result of slower biomass growth rates in the former compared with the second. This can be

considered expected due to the fact that in high oxygenic conditions the complex organic molecules present in MWW are faster oxidized and transformed into simpler carbon sources suitable for bacteria.

3.3. Identification of paracetamol metabolic products

Due to the fact that the paracetamol secondary metabolites can be persistent and more toxic than the drug itself it is essential to verify their fate in biological systems (Wu *et al*. 2012; Marchlewicz *et al*. 2015; Peake *et al*. 2016; Torun *et al*. 2015; Mbokou *et al*. 2016). The major route for biodegradation of paracetamol in microbes was proposed to yield 4-aminophenol and hydroquinone as main intermediates before ring fission and subsequent total mineralization (Wu *et al*. 2012; Zhang *et al*. 2013). Moreover, these two metabolites, as well as 1,4-benzoquinone (the oxidized form of hydroquinone), 4-nitrophenol and NAPQI (N-acetyl-benzoquinone imine) have been reported as intermediates/oxidation products in non-biological advanced oxidation processes (Bedner *et al*. 2006; Moctezuma *et al*. 2012; Postigo and Richardson, 2014).

In this experiment, standards of 4-aminophenol, hydroquinone and 4-nitrophenol were chosen to be used as references for the identification of intermediate products of paracetamol degradation by HPLC analysis. Samples from the experiments with MSM and MWW spiked with paracetamol using sludge from the WWTP oxidation ditch as inoculum revealed high paracetamol removal when the HPLC analysis was conducted using the fast method in isocratic conditions. Therefore, these samples were re-analyzed using an HPLC gradient method that allows a good separation of the metabolites listed above, usually associated to paracetamol degradation (both HPLC methods are described in materials and methods). Two peaks with retention times (RT) corresponding to 4-aminophenol and hydroquinone standards, and another with a RT not matching any of the tested standards (herein referred to as unknown) were detected in samples from the tests in MWW and MSM inoculated with sludge (**Figure 3.4**).

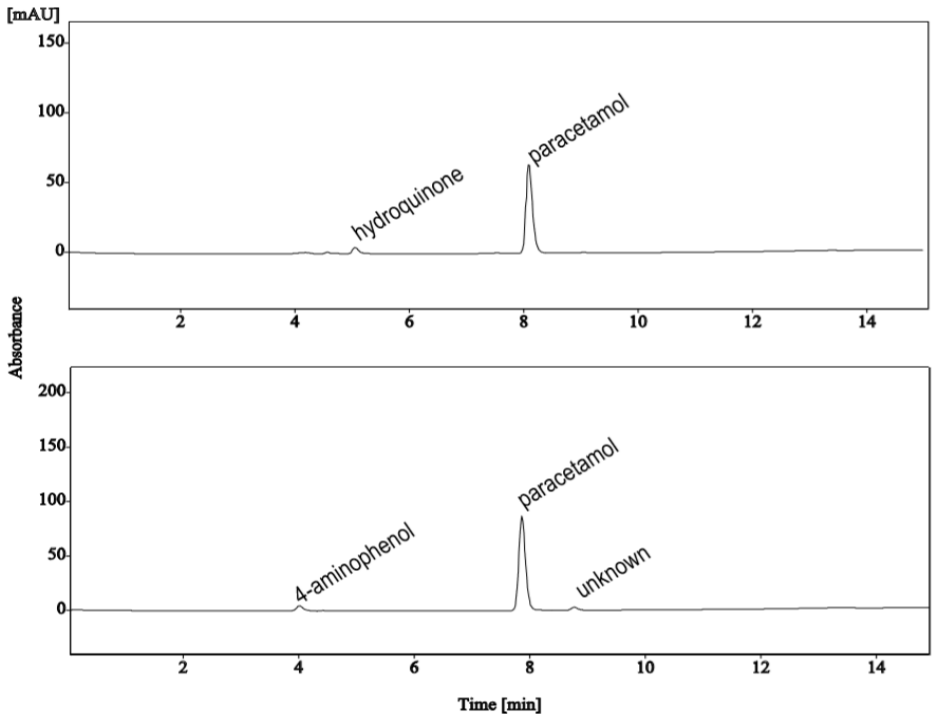

Figure 3.4 Chromatograms obtained by HPLC exemplifying the identification of putative paracetamol biodegradation products in MWW (up) and MSM (down) inoculated with sludge from the WWTP oxidation ditch and containing 50 mg/L paracetamol, collected after two days incubation under aerobic conditions. The retention times are approximately: 4.1, 5.3, 8.0 and 8.8 min for 4-aminophenol, hydroquinone, paracetamol and an unknown product, respectively. Vertical axis = Area (mAUs); horizontal axis = Time (minutes)

The two peaks corresponding to the paracetamol secondary metabolites 4-aminophenol and hydroquinone, as well as the unknown peak, emerged only in samples from cultures in which the concentration of paracetamol decreased. Moreover, they were neither detected at the beginning of the experiment nor in samples from the respective negative controls (without inoculum). This clearly indicates that biological activity is related to the appearance of these metabolites and that they are products from the degradation of paracetamol.

The peak corresponding to 4-aminophenol (RT = 4.1 min) and the unknown peak (RT = 8.8 min) were detected in all sampling days (**Figure 3.5**), whereas the peak corresponding to hydroquinone (RT = 5.3 min) was only occasionally detected with relatively small peak areas: an area of 40.7 ± 3.0 mAUs was detected just in samples from tests with MWW inoculated with aerobic sludge and spiked with paracetamol collected after two days incubation under moderately oxygenated conditions.

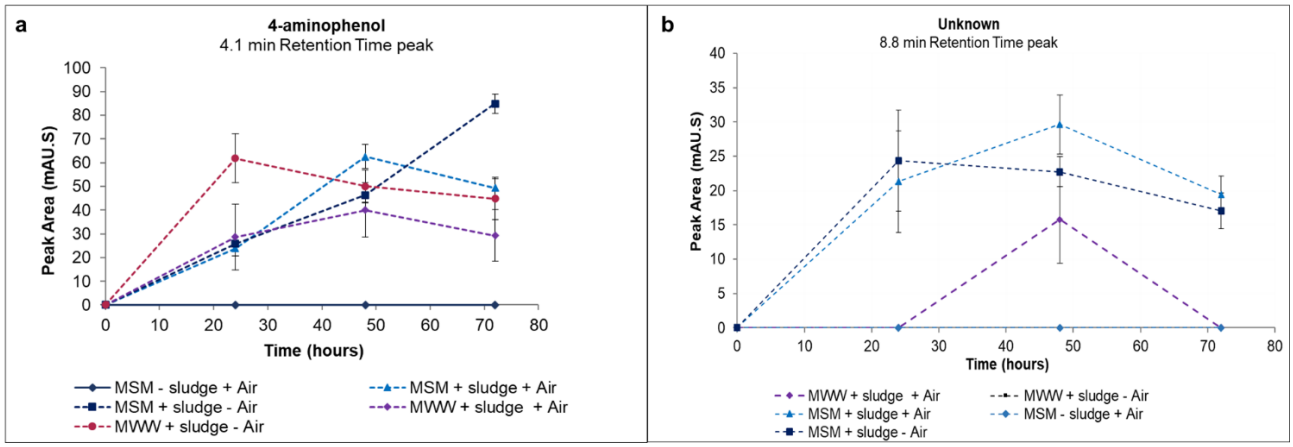

Figure 3.5 Profiles of 4-aminophenol (a) and one unknown metabolite (b), the two putative products from paracetamol biodegradation detected throughout the experiments with MSM and MWW using sludge from the WWTP oxidation ditch as inoculum. Inoculated cultures (+ sludge), negative control without inoculum (– sludge), aerated cultures (+ air) and open flasks cultures without aeration (– air). Values are expressed as the mean ± standard deviation (n=3). In some points the bars are smaller than the symbol, therefore not visible

The occurrence of 4-aminophenol in all inoculated cultures and not in the negative controls (without sludge as inoculum) supports the major biodegradation pathway of paracetamol proposed for microbes by other authors: this drug is metabolized to produce 4-aminophenol, which is converted to hydroquinone through replacement of the amino group by a hydroxyl group, being then this aromatic compound the precursor of several carboxylic acids (2-hexenoic acid, succinic acid, malonic acid, oxalic acid and finally formic acid) (Hu *et al*. 2013; Wu *et al*. 2012; Zhang *et al*. 2013). The rare detection of hydroquinone in these experiments can be due to its fast degradation into those simpler molecules, or because its main production occurred in a later phase of the culture through the biodegradation of 4-aminophenol.

3.4. Effect of paracetamol on COD degradation

COD is an important water quality parameter providing an index to determine the effect an effluent will have on the receiving water body. It is therefore a major reference in the control of wastewater discharges. For example, in Portugal the general COD emission limit value for wastewater discharges is 150 mg O_2/L (Decree-Law nº 236/98 of 1 August) and the limit for discharges from urban WWTP is 125 mg O_2/L (Decree-Law nº 152/97 of 19 June).

In this study the effect of paracetamol on the removal of COD was also addressed. COD was measured in the biodegradation experiments with MWW and MSM using the sludge from the WWTP oxidation ditch as inoculum in the beginning of incubation and after 72 h (**Figure 3.6**).

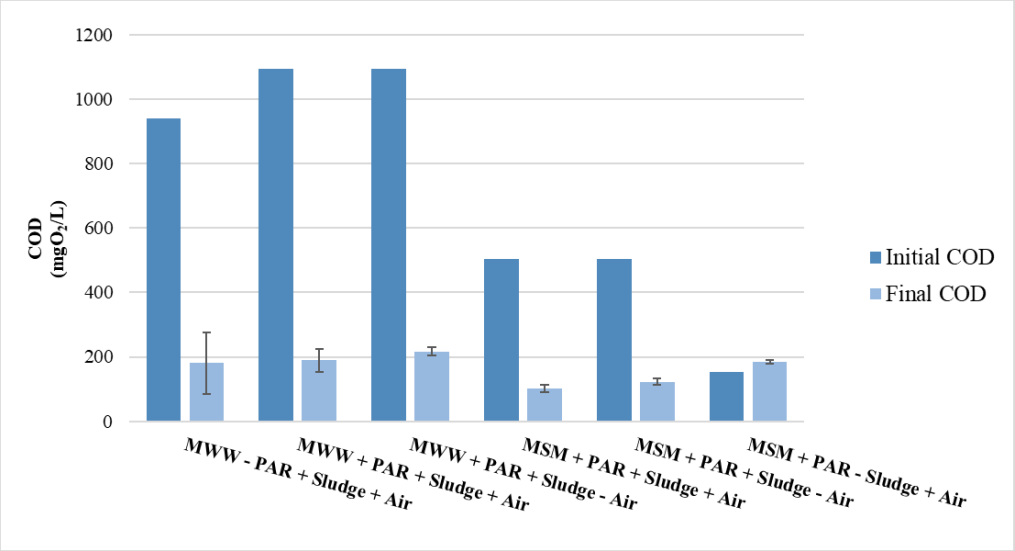

Figure 3.6 COD values measured in the experiments with MWW and MSM using the sludge from the WWTP oxidation ditch as inoculum. Initial and final values were obtained just after inoculation and after 72 h of incubation, respectively. Medium with 50 mg/L paracetamol (+ PAR), medium without paracetamol (- PAR) inoculated cultures (+ sludge), negative control without inoculum (– sludge), aerated cultures (+ air), cultures in open flasks but without aeration (– air). Final values are expressed as the mean ± standard deviation (n=3)

The COD for MWW inoculated with sludge was 940 mg O_2/L, while after addition of 50 mg/L paracetamol was 1095 mg O_2/L. In MSM inoculated with sludge and with 50 mg/L paracetamol COD was 503 mg O_2/L, while in the absence of sludge it was 154 mg O_2/L. This led to the following observations: (i) the tested wastewater had a COD of approximately 590 mg O_2/L, (ii) the sludge inoculum contributed to an increase of approximately 350 mg O_2/L of COD, and (iii) a paracetamol concentration of 50 mg/L generates approximately a COD value of 155 mg O_2/L. After 72 h of incubation the COD values dropped to about 20% of the initial values in both media (MWW and MSM) inoculated with sludge, independently of the presence or absence of 50 mg/L paracetamol, which suggests that the degradation of organic matter achieved with aerobic sludge was not affected by the presence of paracetamol up to this concentration (50 mg/L). This was expected as the IC_{50} values of this drug for bacterial growth, estimated in this work, are around 6 g/L. In contrast, for MSM with 50 mg/L of paracetamol but without sludge inoculum, the COD values after 72 h remained equal to their initial values, confirming the results which showed no removal of paracetamol in this negative control. The final COD values in the tests with MWW varied between 116 and 290 mg O_2/L, with averages around 200 mg O_2/L, which is above the regulatory limit of 125 mg O_2/L for discharges from urban WWTP (Decree-Law nº 152/97 of 19 June). This can be justified with the presence of slow degradable organic compounds from the MWW which are still in solution after 72 h incubation, together with the intermediate metabolites

generated from paracetamol degradation, such as the 4-aminophenol and the unknown compound detected in cultures.

3.5. Bacterial communities degrading paracetamol

Aiming to identify bacteria able to degrade paracetamol, thus with potential for biotechnological applications to improve the removal of this drug from wastewaters, microbial communities were studied on samples from the experiment with aerated cultures of inoculated MSM spiked with 50 mg/L paracetamol, in which high removal rates of this drug were observed. The three replicate cultures of this test revealed identical behaviors for the concentrations of the analyzed compounds along the experiment. Thus, it was considered that the microbial dynamics studied through massive sequencing of 16S rRNA genes present in just one of the replicates along the incubation time would provide enough robust evidence to identify taxa putatively involved in the degradation of paracetamol and/or its metabolites. The study was focused on bacteria using primers for the 16S rRNA gene region V1-3. Samples for microbial community analysis were collected from the WWTP oxidation ditch sludge used as inoculum and from cultures with 1, 2, 3 and 6 days of incubation after inoculation.

DNA sequencing of 16S rRNA amplicons targeting the V1-V3 variable regions was used for taxonomic classifications and to count the numbers of 16S rRNA genes in the samples, which were then used to estimate the relative abundances of bacteria (**Table 3.2**). The number of reads analyzed on each sample (from 66927 to 106904) allowed following the evolution of main taxa from the inoculum towards new dynamic equilibriums (probably with mutualistic relationships) in a medium with paracetamol as the only source of energy (**Table S1**; **Annex 1**).

Table 3.2 The 25 most abundant bacteria (in percent) in samples collected along the experiment with aerated cultures using MSM spiked with 50 mg/L paracetamol inoculated with 10% (v/v) sludge from the Faro Northwest WWTP's oxidation ditch. Each bacterium has both a broad group name (Phylum) and more specific names (Genus and/or family)

			OTU abundances (%)				
Phylum	Family	Genus	Inoculum – sludge	MSM Day 1	MSM Day 2	MSM Day 3	MSM Day 6
Proteobacteria	*Pseudomonadaceae*	*Pseudomonas*	**0.11**	**0.06**	**0.10**	**7.86**	**21.2**
Bacteroidetes	*Flavobacteriaceae*	*Flavobacterium*	**1.66**	**2.52**	**2.57**	**3.67**	**6.91**
Proteobacteria	*Comamonadaceae*		**2.86**	**5.28**	**4.68**	**3.55**	**2.68**
Bacteroidetes	*Chitinophagaceae*	*PHOS-HE31*	**2.16**	**3.31**	**4.10**	**3.37**	**2.35**
Bacteroidetes	*PHOS-HE51*		**2.25**	**2.63**	**3.62**	**3.91**	**2.07**
Proteobacteria	*Xanthomonadaceae*	*Dokdonella*	**1.48**	**2.20**	**3.13**	**3.29**	**3.80**
Bacteroidetes	*Saprospiraceae*	*QEDR3BF09*	**6.06**	**2.34**	**1.81**	**1.02**	**0.87**
Bacteroidetes	*Saprospiraceae*	*CYCU-0281*	**3.36**	**2.81**	**2.71**	**1.15**	**1.03**
Proteobacteria	*Rhodocyclaceae*	*Azospira*	**6.03**	**1.26**	**1.29**	**0.81**	**1.52**
Proteobacteria	*Rhodocyclaceae*	*Uliginosibacterium*	**2.67**	**3.43**	**2.13**	**1.96**	**0.23**
Proteobacteria	*Comamonadaceae*	*188up*	**2.81**	**3.30**	**2.55**	**1.00**	**0.62**
Proteobacteria	*Comamonadaceae*	*Comamonas*	**0.44**	**0.12**	**0.15**	**0.10**	**0.20**
Bacteroidetes	*NS9 Marine group*	*PHOS-HE28*	**1.63**	**1.89**	**2.40**	**1.65**	**1.61**
Bacteroidetes	*Saprospiraceae*	*MK04*	**2.42**	**2.20**	**2.14**	**0.64**	**1.16**
Proteobacteria	*Rhodocyclaceae*	*Dechloromonas*	**0.92**	**1.35**	**0.97**	**2.15**	**1.94**
Proteobacteria	*Methylophilaceae*	*Methylophilus*	**0.00**	**0.08**	**0.34**	**0.43**	**3.83**
Proteobacteria	*Alcaligenaceae*	*Achromobacter*	**0.00**	**0.00**	**0.00**	**0.00**	**0.05**
Proteobacteria	*Hyphomicrobiaceae*	*Hyphomicrobium*	**0.40**	**2.10**	**2.07**	**1.00**	**1.63**
Proteobacteria	*Comamonadaceae*	*Acidovorax*	**0.75**	**1.18**	**1.05**	**1.18**	**1.74**
Proteobacteria	*Campylobacteraceae*	*Arcobacter*	**2.33**	**0.86**	**0.84**	**1.62**	**0.24**
Proteobacteria	*Burkholderiaceae*	*Lautropia*	**0.58**	**1.19**	**1.52**	**0.91**	**1.55**
Chloroflexi	*Anaerolineaceae*	*SBR1029*	**0.26**	**1.90**	**1.60**	**0.71**	**0.21**
Bacteroidetes	*Cytophagaceae*	*uncultured*	**0.28**	**0.79**	**0.97**	**1.18**	**1.73**
Proteobacteria	*Rickettsiales Incertae Sedis*	*Candidatus Odyssella*	**0.15**	**0.85**	**1.15**	**0.67**	**1.94**
Verrucomicrobia	*Opitutaceae*	*Opitutus*	**0.69**	**1.05**	**1.07**	**1.50**	**0.47**

This evolution in the diversity seems to reflect shifts in the bacterial population caused by a trend towards the extinction of strains not capable of degrading paracetamol or the intermediates of its degradation and the survival of strains putatively with that capacity.

Within the 25 most abundant bacterial groups, the gradual decreases in percentages of 16S rRNA gene amplicons from genera *QEDR3BF09*, *CYCU-0281*, *Azospira*, *Comamonas*, *MK04* and *Arcobacter* over the incubation time of the experiment suggest that the representatives of these genera in this population were not able to use paracetamol, or the products of their degradation as sources of energy. On the other hand, the percentages of 16s rRNA gene amplicons classified in the genera *PHOS-HE31*, *Uliginosibacterium*, *188up*, *PHOS-HE28*, *Hyphomicrobium*, *SBR1029*, *Candidatus Odyssella*, *Opitutus* and in two groups from families *Comamonadaceae* and *PHOS-*

HE51 not classified by genus, showed a rising trend followed by decline throughout the experiment (**Table 3.2**). These amplicons may be from bacteria that have used as nutrients the products generated by the degradation of other microorganisms in the culture which became perishable as the conditions changed ceasing to be viable for them.

Finally, the continuing raise of 16S rRNA gene amplicons relative abundances from some taxonomic groups along the whole experiment (genera *Pseudomonas*, *Flavobacterium*, *Dokdonella*, *Dechloromonas*, *Methylophilus*, *Achromobacter*, *Acidovorax*, *Lautropia* and a group of uncultured bacteria from family *Cytophagaceae*) indicates the presence of bacterial strains from these groups probably capable of metabolizing paracetamol and/or its degradation intermediates to obtain energy. Among these, the genera *Pseudomonas*, *Flavobacterium*, *Dokdonella* and *Methylophilus* were in evidence with final abundances of 21.2%, 6.91%, 3.8% and 3.83%, respectively.

Within the genus *Pseudomonas*, OTU_7 (GenBank accession MG554751) stands out with an increase from the inoculum to the sixth day from 0.04% to 20.63%, while for the other seven OTUs of this genus there were no increases or increases were less than 0.3% (**Table S1; Annex 1**). For OTU_7 the highest increases were from the second to the third day, *i.e.* when the largest decrease in paracetamol concentration occurred (**Figure 3.3**: MSM + sludge + air) and between the third and the sixth day, *i.e.* when there was no paracetamol but intermediate products of its degradation were available, such as those analyzed in this work: 4-aminophenol and an unidentified product (**Figure 3.5**: MSM + sludge + air). This suggests that, most probably, the bacteria corresponding to OTU _7 had an important contribution both in the first phase of paracetamol biodegradation and in the subsequent phase of biodegradation of the products generated in the first phase. These results together with other results previously reported (Khan *et al*. 2006; De Gusseme *et al*. 2011; Zhang *et al*. 2013; Karaman *et al*. 2016) reinforce the idea that some *Pseudomonas* strains have the ability to metabolize paracetamol and the intermediate of its degradation 4-aminophenol as the sole source of energy. With the RDP classifier (Wang *et al*. 2007) the classifications were just up to the genus (**Table 3.2**), so for OTUs putatively corresponding to bacteria with a role in the degradation of paracetamol an attempt was made to classify the species by alignment with BLASTN (Zhang *et al*. 2000) in the database rRNA_typestrains/prokaryotic_16S_ribosomal_RNA. However, it was not possible to point out a more probable species to which OTU_7 corresponded because its alignment revealed sequences of 8 different species of *Pseudomonas* with more than 99% coverage (query cover) and 99% similarity (identities) (*P. umsongensis, P. helmanticensis, P. taiwanensis, P. vancouverensis, P. graminis, P. entomofila, P. monteilii, P. lutea*), as well as a series of sequences of other species of this genus fully aligned and with similarities of 98%. Curiously, among these there was no sequence of *P. aeruginosa*, a species identified by other authors as having the ability

to degrade paracetamol (De Gusseme *et al*. 2011; Karaman *et al*. 2016). Thus, the results here presented suggest that other species of this genus may have also that ability.

In the genus *Flavobacterium*, 47 OTUs were identified, but only one (OTU_20; GenBank accession MG554764) was noted for percentage increase over the experiment, while in the genus *Methylophilus* only one OTU was identified (OTU_23; GenBank accession MG554767). In these cases, the highest increases in the percentage occurred after the third day, *i.e.* when paracetamol was not available but only metabolites of its degradation. Trends in decreasing concentrations of 4-aminophenol and the unidentified product observed from the second to the third day of incubation (**Figure 3.5**: MSM + sludge + air) indicate the beginning of a phase of the culture in which these products began to be degraded due to the proliferation of bacteria capable of metabolizing them.

This is not the first time that microbial populations exposed to a pharmaceutical led to shifts towards an evident selection of *Flavobacterium* species. For example, in cultures acclimated with the antibiotic enrofloxacin a selection of the genera *Flavobacterium* was observed with its relative abundance increasing 20.8% (Alexandrino *et al*. 2017). In fact, by the end of the 70's it had already been described the isolation of a *Flavobacterium sp*. capable of degrading the herbicide asulam and its structure analogous sulphanilamide in a synthetic medium with no other carbon sources added (Walker, 1978). Then, the isolation of plasmids carrying genes for degradation of aromatic compounds (Chaudhry and Huang, 1988) and the purification of enzymes capable of hydrolyzing aromatic compounds (Van Berkel and Van Den Tweel, 1991) from *Flavobacterium* strains, achieved in the subsequent years, clearly showed the potential biotechnological application of those strains for the bioremediation of recalcitrant pollutants. The isolation of *Flavobacterium* strains biodegrading nylon oligomers (Kinoshita *et al*. 1981) is another evidence of the capacity of these microorganisms to use synthetic recalcitrant compounds as the sole source of carbon. The capacity of biodegrading nylon oligomers was also identified on *Pseudomonas* strains (Kanagawa *et al*. 1993), which is a clue for the possibility of common metabolic pathways in these two taxonomic groups. A *Flavobacterium sp.* able to use 4-nitrophenol as a carbon and energy source has already been isolated (Raymond and Alexander, 1971) and this compound could also be used as the sole carbon and energy source by a strain of *P. aeruginosa* (Zheng *et al*. 2009), a specie with strains able to biodegrade paracetamol and 4-aminophenol as described above. Therefore, based on the results here presented it seems reasonable to propose that probably the *Flavobacterium* strains in the culture corresponding to OUT_20 was capable of biodegrading 4-aminophenol. The alignment of OTU_20 in the NCBI database revealed a *Flavobacterium luvivivi* sequence with 100% coverage and 99% similarity and sequences of several species of this genus with similarities of less than 95%.

An association between *Methylophilus* and successful biodegradation of pharmaceuticals using next-generation sequencing of 16S rRNA genes over time, besides the work here presented, was also described recently by Kim *et al*. (2017), though not for paracetamol. Evidence that *Methylophilus* can grow on methylated amines (mono-, di- and trimethylamine) have been reported (Large and Haywood, 1981). However, this genus is known as a group of methanol-utilizing bacteria (Jenkins *et al*. 1987) and methanotrophs have been studied for their potential to be used directly in bioremediation due to the methane monooxygenase enzyme(s) they possess, which have broad substrate specificity and have been shown to co-oxidise aromatic pollutants (De Marco *et al*. 2004). For example, a *Methylophilus sp.* isolated from a humic lake degraded phenol and humic matter (Hutalle-Schmelzer *et al*. 2010). These evidence together with the results of this work support the hypothesis that the *Methylophilus* bacteria corresponding to OTU_23 was also able to metabolize products resulting from the degradation of paracetamol, either the aromatic compounds generated in the first steps of the degradation of this drug, or the simpler compounds subsequently generated. The alignment of OTU_23 in the NCBI database revealed sequences from several *Methylophilus* species with coverages and similarities of less than 97% and two sequences with 98% coverage and 99% similarity, one from *M. methylotrophus* and one from *M. leisingeri*.

In the genus *Dokdonella* 5 OTUs were identified, but also in this case only one (OTU_10; GenBank accession MG554754) stood out in the evolution of relative abundances. The percentage of amplicons in this OTU increased mainly in the first two days of incubation, then remained almost the same from the second to the third day and after six days it increased again. Though the genus *Dokdonella* has not yet been associated with pharmaceutical degradation it has been detected in alkane degrading cultures (Alonso-Gutiérrez *et al*. 2009) and a *Dokdonella* potentially degrading polycyclic aromatic hydrocarbons was detected by Bacosa and Inoue (2015). Moreover, *Dokdonella* has been previously isolated from denitrifying environments in the presence of O_2 (Sun *et al*. 2009) and was one of the predominant microorganisms in a study with activated sludge at 2% O_2 concentration (without inhibitory effects on N_2O biodegradation), in which it was suggested that heterotrophic denitrification was the most likely mechanism of N_2O removal (Figueroa-González *et al*. 2016). It may therefore be hypothesized that the *Dokdonella* corresponding to OTU_10 may have used the nitrate generated after the release of the amine group from 4-aminophenol (by substitution by a hydroxyl group) as an electron receptor to obtain energy by oxidation of hydroquinone (the aromatic organic compound generated by the amine group release of 4-aminophenol) and/or by oxidation of the simpler carbon compounds generated in the degradation of hydroquinone by other microorganisms (such as *Pseudomonas*). The alignment of OTU_10 in the NCBI database revealed a *Dokdonella immobilis* sequence with 100% coverage and 94% similarity and sequences of several species of different genera with similarities of 92% or less.

4. CONCLUSIONS

Estimated paracetamol IC_{50} values for bacterial growth under anaerobic and aerobic conditions are similar (6.204 and 6.162 g/L, respectively) and much higher than the concentrations of this drug usually detected in WWTP influents and effluents.

In what concerns the biodegradation of paracetamol, the results indicate that aerobic microorganisms had a major role in the degradation of this drug. In municipal wastewater inoculated with sludge from a WWTP's oxidation ditch paracetamol (50 mg/L) was removed below the limit of detection, after two days incubation in batches with aeration and after three days in open batches without aeration. The metabolites 4-aminophenol and hydroquinone, two known intermediates of paracetamol degradation, plus one compound not identified in this work were produced in the cultures during paracetamol degradation. Moreover, it was observed that 50 mg/L of paracetamol generates a COD of about 155 mg O_2/L and that such a concentration of this drug does not affect the degradation of COD in municipal wastewater with activated sludge-based treatments.

Regarding the identification of bacteria with a role in the degradation of paracetamol, this study corroborates previous works reported in literature showing the ability of species from *Pseudomonas* genus to use this drug and intermediates of its degradation as sources of energy and hypothesizes for the first time that species from genera *Flavobacterium*, *Dokdonella* and *Methylophilus* may have the capacity to degrade the metabolites produced from paracetamol's degradation.

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CHAPTER 4

Biodegradation of paracetamol by bacterial isolates assigned to species of *Bacillus cereus* group, *Corynebacterium nuruki*, *[Brevibacterium] frigoritolerans* and *Enterococcus faecium*

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ABSTRACT

Bacterial isolates with capacity to degrade paracetamol were obtained from an activated sludge sample collected in an oxidation ditch of a wastewater treatment plant. Among these twelve bacterial isolates were selected based on their ability to grow in the presence of paracetamol. They were identified using the colony morphotype procedure and by 16S rRNA gene sequencing analysis, but only four of them showed ability to use paracetamol as the sole carbon source in the presence of a nitrogen supply. Those four bacterial isolates were assigned to species of the genera *Bacillus*, *[Brevibacterium]*, *Corynebacterium* and *Enterococcus*. Bacterial isolates were cultured in liquid MSM spiked with 200 mg/L and 500 mg/L of paracetamol at 28 ºC in dark. *[Brevibacterium] frigoritolerans*, *Corynebacterium nuruki* and *Enterococcus faecium* removed 97 ± 4%, 97 ± 6% and 86.9 ± 0.8% of paracetamol at 200 mg/L, respectively, while a species belonging to *Bacillus cereus* group (*Bacillus pacificus* strain MCCC 1A06182 or *Bacillus paranthracis* strain MCCC 1A00395) removed the drug below the limits of detection with evidence of mineralization, after 144 h of incubation. When the concentration of paracetamol increased to 500 mg/L, the cultures of "*Bacillus cereus* group", *[Brevibacterium] frigoritolerans* and *Corynebacterium nuruki* remained able to remove 95 ± 5%, 95 ± 8% and 95 ± 4% of the drug, respectively. During the degradation process, the metabolites 4-aminophenol, hydroquinone and 2-hexenoic acid were detected. To the best of our knowledge these species are reported for the first time as paracetamol degraders.

Keywords Bioremediation, Bacterial isolates, Paracetamol, Secondary metabolites

1. INTRODUCTION

In the recent years, many pharmaceutical residues have been detected in the aquatic environments (Winker *et al*. 2008; Kümmerer, 2009) contaminating sewage and wastewater effluents in the proximity of wastewater treatment plants (WWTPs), surface and ground water, and drinking water (Ganiyat 2008; Yang *et al*. 2008; Brandão *et al*. 2014; Cardoso *et al.* 2014). Pharmaceuticals commonly found in aquatic environments included antibiotics, anticonvulsants, antipyretics, cytostatic drugs, and hormones. These compounds reach water bodies through various pathways, such as direct disposal of expired and remnant domestic drugs, excretion from human body by in faeces/urine of parent chemicals with several associated metabolites after therapeutic use, and inadequate treatment of effluents from the manufacturing industry (Ganiyat, 2008; Yang *et al.* 2008; Petrie, Barden and Kasprzyk-Hordern, 2015).

As a consequence of their continuous use by human populations these substances have a widespread environmental distribution. Pharmaceuticals possess biological activity being particularly designed to resist metabolic degradation, having in addition, a lipophilic structure which is generally a basic requirement to maximize absorption by target organisms. These characteristics must be considered when evaluating the drugs toxicological impact in the wild (Wils *et al*. 1994; Halling-Sørensen *et al*. 1998; Daughton and Ternes 1999; Miao *et al*. 2002; Jones *et al*. 2005(a, b); Shaji and Patole, 2008; Snape *et al.* 2010; Brandão *et al*. 2014; Singh, 2017). Also, several metabolites may be formed, and toxicological interactions would very likely occur among pharmaceutical residues in the environment (Cleuvers, 2003, Brandão *et al*. 2014). Therefore, most of these xenobiotics may be considered as active, effective, recalcitrant environmental contaminants and some of them are biomagnified to dangerous/toxic levels (Jha *et al.* 2015). One of the most extensively used drug worldwide is paracetamol (N-acetyl-p-aminophenol), also known as acetaminophen (Malar and Bai, 2012; Jóźwiak-Bebenista and Nowak, 2014; Kar *et al*. 2015).

Paracetamol is considered a safe non-steroidal anti-inflammatory drug (NSAID) at therapeutic doses, which is widely used in the treatment of pain and fever and provides an effective relief of these symptoms (Xu *et al*. 2008).

In the human liver, paracetamol is metabolized to the non-toxic conjugated metabolites acetaminophen glucuronide and acetaminophen sulphate. Under normal conditions, less than 10% of the paracetamol is metabolized by the cytochrome P450 enzymes (primarily CYP 2E1, 1A2, and 3A4) to produce a toxic metabolic intermediate called N-acetyl-p-benzoquinoemine (NAPQI). The small amount of NAPQI formed is normally further detoxified by intracellular glutathione (Xu *et al*. 2008).

Univar estimates that 115000 tons of paracetamol are consumed each year, with 25% of production going to Europe (Pharmafile, 2009).

Despite its common use, there is very limited information on paracetamol concentrations in municipal wastewater (Vymazal *et al*. 2017). Drug concentrations varied from 350 to 180000 ng/L and 10 to 13000 ng/L in the inflowing and outflowing wastewaters among the surveyed systems in Czech Republic´s constructed wetlands (Vymazal *et al*. 2017). Kasprzyk-Hordern *et al*. (2009) reported paracetamol concentrations ranging from 1826 to 24525 ng/L in the effluents of WWTP's Cilfynydd (South Wales) (Kasprzyk-Hordern *et al*. 2009; Vymazal *et al*. 2017), whereas in Volos´s WWTP, Greece, the concentrations of paracetamol in the effluents of aerobic tanks systems were up to 305 ng/L (Papageorgiou *et al*. 2016). Paracetamol was found at concentrations up to 6 μg/L in European WWTP effluents (Ternes, 1998), although in Portugal, the highest concentration of paracetamol recorded in wastewater effluent was 32 μg/L (Pereira *et al*. 2016). In fact, the worldwide presence of this drug in the WWTP effluents reminds the need to implement more efficient treatment processes for its removal, even if in general the higher concentrations of paracetamol detected in raw sewage than in treated wastewaters indicate high removal rates (*e.g.* Roberts and Thomas, 2006; Papageorgiou *et al*. 2016). Though paracetamol appears to be well removed during sewage treatment when compared to other pharmaceuticals, it was detected in surface waters ranging from 110 to 10000 ng/L (Wilkinson *et al*. 2017), *e.g.* in the river Taff, United Kingdom was found in concentrations from 305 to 1534 ng/L (Kasprzyk-Hordern *et al*. 2009) and in USA's natural waters at concentrations up to 10 μg/L (Jallouli *et al*. 2017). Also, Olaitan *et al*. (2014) reported of paracetamol in an average concentration of 3 μg/L in surface and groundwater samples collected from an irrigation canal and several wells in a pharmaceutical industrial area of Sango Ota, Ogun State, Nigeria. Additionally, during a national survey to trace organic contaminants in Australian rivers, paracetamol was one of the most detected contaminants at maximum concentrations of 7150 ng/L (Scott *et al*. 2014). Another sign that this pollutant should be of global concern is paracetamol's detection in the open sea waters of the western Mediterranean at concentrations ranging from 0.468 to 1.70 ng/L (Brumovský *et al*. 2017; Palma *et al*. 2018).

The detection of paracetamol and its metabolites in surface water suggests that the degree of human use and the disposal in sewage overwhelms their effective removal by conventional sewage treatment (Peake *et al*. 2015). Given that metabolites can be persistent, and some are even more toxic than the parent compound, it is essential to determine their fate in biological systems (Wu *et al*. 2012; Marchlewicz *et al*. 2015; Peake *et al*. 2015; Torun *et al*. 2015; Mbokou *et al*. 2016) and, hence, to search for new and effective ways to degrade or remove them from wastewater and resulting treated effluents (Palma *et al*. 2018).

The cost of chemical agents, the energy source, and the generation of secondary pollutants due to the use of excessive chemicals are the major drawbacks by using (photo)chemical methods on removing the paracetamol from wastewaters (Trovo *et al*. 2008; Abdullah *et al*. 2018). In this

regard, biodegradation may represent an environmentally friendly and low-cost effective solution (Wu *et al*. 2012).

Microorganisms, particularly bacteria, have been demonstrated to play an important role in the biodegradation of organic compounds in WWTPs (Pieper and Reineke, 2000). In WWTP processes, biodegradation can be partial or complete. When biodegradation is complete, the substances (*e.g.* drugs) are converted to inorganic substances, such as water and carbon which is converted to carbon dioxide in a process called mineralization, whereas the partial breakdown of the drugs results in their transformation into metabolites (Alexander, 1999).

Singh (2017) named some important bacteria for drugs biodegradation processes in which are included *Bacillus* (capable of producing thick-walled endospores that are resistant to heat, radiation and chemical disinfection), *Pseudomonas*, *Klebsiella*, *Actinomycetes*, *Nocardia*, *Streptomyces*, *Thermoactinomycetes*, *Micromonospora*, *Mycobacterium*, *Rhodococcus*, *Flavobacterium*, *Comamonas*, *Escherichia*, *Azotobacter* and *Alcaligenes*.

In the particular case of paracetamol, several microorganisms have been isolated and found to be capable of using this drug as a carbon and energy source; metabolic pathways for the biodegradation of this drug have been proposed (Neumann *et al*. 2004; Cámara *et al*. 2009; De Gusseme *et al*. 2011; Zhang *et al*. 2013; Karaman *et al*. 2016). De Gusseme *et al*. (2011) reported two paracetamol degrading strains, *Delftia tsuruhatensis* and *Pseudomonas aeruginosa,* that were isolated from a membrane bioreactor (MBR) inoculated with an enriched nitrifying bacterial culture, which were able to remove continuously 99.9% of 100 μg/L of the drug. During the incubation of these isolates, hydroquinone was formed and considered a potential transformation product. Furthermore, Zhang *et al*. (2013) have isolated from microbial aggregates three bacterial strains of the genera *Stenotrophomonas* and *Pseudomonas* capable of using paracetamol as their sole carbon, nitrogen and energy sources and proposed metabolic pathways for the degradation of this drug, with 4-aminophenol and hydroquinone as first metabolic products. Karaman *et al*. (2016) demonstrated that paracetamol in activated sludge underwent biodegradation within less than one month and reported that *P. aeruginosa* was the responsible for the biodegradation of this pharmaceutical to 4-aminophenol and hydroquinone. In fact, *Pseudomonas* is also known for its ability to degrade other aromatic compounds of environmental concern (Cámara *et al*. 2009; Neumann *et al*. 2004). More recently, Abdullah *et al*. (2018) found *Pseudomonas aeruginosa* strains signed as STB2 and STB4 with ability to degrade 79.4% and 88.4% of 3000 mg/L of paracetamol, respectively, in 120 h.

The present study focuses on the search for bacterial isolates with capacity to biodegrade paracetamol. For that purpose, the bacteria were isolated from a sludge containing an aerobic

community, which were used in previous studies performed by our research group (Palma *et al*. 2018), that proved to biodegrade paracetamol.

2. MATERIAL AND METHODS

2.1. Source of inoculum and culture preparation

For this study a sample of aerobic activated sludge was collected from the oxidation ditch of Faro Northwest WWTP's, Portugal, to test the paracetamol biodegradability of by bacterial isolates. This sludge was previously used in studies aiming the selection of an aerobic community with ability to degrade paracetamol (Palma *et al*. 2018).

After collection, 1 g of sludge was rinsed with a Ringer's solution (0.008 g/L NaCl, 0.0003 g/L KCl, 0.00033 g/L $CaCl_2.H_2O$) to eliminate all dissolved carbon compounds to ensure that paracetamol was the only available carbon source for bacterial growth. For this purpose, the inoculum from the aerobic sludge was centrifuged at 2500 ×g for 10 minutes at room temperature, then the supernatant was discarded, and the pellet was subsequently used in successive dilutions. The washing procedure was repeated twice.

2.1.1. Bacterial isolates from an aerobic community

The pellet resulting from the sludge previously washed was incubated in 9 mL of 0.1% peptone water at 28 °C for 2 h. A successive dilutions protocol was performed by adding 1 mL of the original culture in 9 mL of broth to each tube. Subsequently, 100 µL of each dilution was spread onto the solid plate cultures with mineral salt medium (MSM) a medium without carbon compounds which was composed by 0.5 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.01 mg/L NaCl, 0.2 g/L $MgCl_2.6H_2O$, 0.02 g/L $CaCl_2$, 0.0004 mg/L $ZnSO_4.7H_2O$, 0.0004 g/L $CoCl_2.6H_2O$, 0.0003 mg/L $MnSO_4$, 0.01 mg/L of ethylenediamine tetraacetic acid (EDTA) and 0.0003 mg/L $(NH_4)_6Mo_7O_{24}.4H_2O$), with a nitrogen source of 1 g/L $(NH_4)_2HPO_4$ and agar (15 g/L). The experiments were carried out adding to the solid MSM:

i. 10 g/L glucose, as positive control, to monitor bacterial growth, given that this compound is the preferred carbon source of most of the common heterotrophic bacteria (Moat *et al.* 2002);
ii. 200 and 500 mg/L of paracetamol as unique carbon source.

The isolates which grew in the presence of 500 mg/L of paracetamol were cultured at a lower concentration of the drug (200 mg/L).

Two main explanations can be given on why high concentrations of paracetamol, above those found in the environment, were used in the present work. The resistance of bacteria to such high concentrations was studied as well as its biodegradation. In this study paracetamol was used as sole

carbon source, thus the necessity of such high concentrations to ensure that there was enough carbon source to allow the optimal growth of bacteria. Other authors performed their experiments using very high concentrations of paracetamol such as for example: Zhang *et al.* 2012; Zhang *et al.* 2013; Abdullah *et al.* 2018. Also, it can be assumed in this case that if bacteria can resist to and degrade higher concentrations then the residual concentrations would be easily removed from the wastewaters (from ng/L to pg/L) of the drug.

These cultures performed in solid medium allowed to carry out the selection and isolation of bacteria resistant to paracetamol. The bacterial isolates were selected using colony morphotypes, which is defined as a group of bacteria exhibiting typical colonial patterns (pigmentation, shape, size, elevation, margin) (Lebaron *et al*. 1998; Fleury *et al.* 2001). Bacteria belonging to the same morphotype, if under similar growth conditions, develop colonies of the same morphology for certain growth conditions. Colonial growth patterns of a morphotype are inherited and can be generated following inoculation with a single cell (Fleury *et al.* 2001). The selected colonies with ability to grow in the presence of paracetamol were picked and purified for further identification and to perform paracetamol biodegradation experiments.

Catalase activity was determined by observing bubble production after the application of 3% (v/v) hydrogen peroxide solution, a positive reaction is indicated by the production of bubbles. Oxidase activity was evaluated with Microbact oxidase strips (OXOID).

The gram staining protocol was accomplished as described by Gram 1884 and observed on a light microscope (Leica Microsystems).

2.2. Identification of isolates that degrade paracetamol

2.2.1. Biomass production for DNA extraction

From a culture grown in the presence of 500 mg/L of paracetamol, a colony was picked up with a wire loop and re-inoculated using the streak plate technique on solid MSM with 200 mg/L and 500 mg/L of the drug, respectively, in order to test the bacterial resistance over generations. The bacterial isolates were maintained and stored as aqueous glycerol suspensions (30%, v/v) at -20 °C.

In order, to identify the microorganisms present in the samples, biomass was produced to guarantee the amount required for DNA extraction. For biomass production, 100 µL of glycerol culture was poured onto solid plate count agar (PCA) for Standard Method Agar provided by HiMedia (USA) growth medium at 23.5 g/L and the spread plate method was performed with a glass spreader. Cultures were incubated at 28 °C for 48 hours. All the assays were performed in the dark to avoid drug's photodegradation.

2.2.2. DNA extraction and amplification of 16S rRNA

To identify bacterial isolates putatively capable of degrading paracetamol, DNA was extracted from each isolate bacterial culture with the PowerSoil® DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), which uses bead beating and silica spin filter technology for extraction. The concentration and quality of eluted DNA was determined using a NanoDrop spectrophotometer Thermo Scientific 3300 for further amplification of the DNA extracted through the polymerase chain reaction (PCR).

The 16S rRNA gene was amplified from the DNA samples extracted from the bacterial isolates. For the PCR amplification of the bacterial 16S rRNA gene conserved region the following universal primers were used the 8F (*forward*) 5'-AGAGTTTGATCCTGGCTCAG-3'/ 1492R (*reverse*) 5'-TACCTTGTTACGACTT-3'.

For the reaction mixture, 2 µL of DNA sample, 1 µL of forward primer, 1 µL of reverse primers (8), 2.5 µL of Dream Taq Buffer, 0.1 µL of Dream Taq DNA polymerase and 0. 5 µL dNTPs mixture (NZytech). For a final volume of 25 µL of reaction.

Amplifications were performed on the thermal cycler 2720 (Applied Biosystems) using the following conditions: initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 1 minute (denaturation), 57 °C for 30 seconds (primer annealing) and 72 °C for 2 minutes (elongation), and final elongation at 72 °C for 5 minutes.

The gel electrophoresis was performed using 5 µL of amplified PCR product to which it was added 2 µL of loading dye buffer x6 (NZYtech) containing blue and orange bromophenol dyes G on an agarose gel (1% agarose, Tris- 1% Acetate-EDTA (TAE)). According to the manufacturer's instructions (Invitrogen), 3 µL of DNA safe SYBR (NZYtech) was added to the agarose. 5 µL of 1 kb NZYDNA Ladder VII (NZYtech) was used as the molecular marker to estimate, by comparison, the size of the DNA fragments analyzed. The migration was performed at 100 V for 1 hour. The gel was examined in a UV transilluminator (Biorad Universal Hood II Gel Doc System, UK) for visualization of the DNA fragment bands.

2.2.3. DNA sequencing

The obtained isolates were further identified by DNA of 16S rRNA genes amplification (originating a purified PCR product of 1484 bp). Direct sequencing of the PCR products was performed by the Sanger method. The sequencing reaction was performed using the purified PCR product (the template amount *per* sequencing reaction for amplifications between 1000 and 2000 bp should be about 40 ng of DNA) corresponding to the amplification of the 16S rRNA gene with *primer*s 8F/1492R and the *BigDye Terminator Cycle sequencing* v*3.*1. kit (Applied Biosystems, USA) (with a labeling dye for each of the nucleotides). The POP-7 polymer (Applied Biosystems, USA) was

used as separation matrix for performing DNA sequencing and fragment analysis in a Genetic Analysis 3130xl sequencer (Applied Biosystems, USA). The results were obtained by Sequencing Genetic Analysis Software, provided by CCMAR's Molecular Biology platform.

The sequence reads analysis were treated using the BIOedit Sequence Alignment Editor and contig-0 sequences were generated as listed in **Table S3**, **Annex 2**. Taxonomic identity was established through Blast search of Gene Bank database of NCBI and RDP Classifier and the Multiple-sequence alignment was performed in MEGA-X version 10.0.5 (Kumar *et al*. 2018) using the ClustalW package. Tamura-Nei model was used as nucleotide substitution method with discrete Gamma distribution (Tamura and Nei 1993), while the phylogenetic tree analysis was performed using the maximum likelihood with 500 bootstrap replications.

2.3. Paracetamol biodegradation assays

The bacteria which grew in the presence of paracetamol were isolated in PCA medium for further use. Liquid cultures were carried out, in order, to study the biodegradation of paracetamol with a dilution of 1:300 (v/v) of each bacterial isolate that displayed the ability to grow in the presence of paracetamol. After bacterial isolates identification, the further experiments were run in liquid medium using the MSM performed as described above supplemented with a nitrogen source of 1 g/L of $(NH_4)_2HPO_4$ and a carbon source: 10 g/L glucose (positive control) and spiked with 200 and 500 mg/L of paracetamol. Negative controls only with the drug and in the absence of the bacterial isolates were performed. The bacterial isolate cultures were incubated at 28 °C and 150 rpm in the dark. The sampling was carried out after 48 h and 144 h, times chosen due to the slow growth presented by these bacteria in the studied conditions. The assays were performed in triplicate. The bacterial growth was measured by optical density (OD) at 600 nm in a Hach-Lange™ DR 2800 spectrophotometer (Sköndal, Sweden).

A preliminary assay was carried out by pooling the bacterial isolates which showed the best performance as paracetamol bio-degraders. The culture was performed by joining each isolate in a dilution of 1:300 (v/v) at 200 mg/L of paracetamol, under the same experimental conditions as described above.

2.3.1. Identification of paracetamol metabolic products by high performance liquid chromatography (HPLC) analysis

Immediately after collecting the samples, they were filtered with 0.2 µm polypropylene (PP) syringe filters from VWR (Leuven, Belgium) and then stored overnight at 4 ºC before chromatographic analysis.

Paracetamol (99% purity), 4-aminophenol (97% purity), hydroquinone (99% purity), benzoquinone (≥98%), 2-hexenoic acid (99%) and 4-nitrophenol (99% purity) used for standard solutions were purchased from Sigma-Aldrich. The concentration range of paracetamol standards was from 1 to 600 mg/L of drug.

The degradation of paracetamol was analyzed, and the products known to be generated from the degradation of this drug were identified using a modular Advanced Scientific Instrument KNAUER HPLC system with a Smartline UV detector 2600 Smartline Manager 5000 (Berlin, Germany). The output signal was monitored and integrated using ClarityChrom® software. The compounds were separated using a reversed phase XbridgeC18 column (4.6 × 250 mm, 5 µm particle size) - Hybrid technology connected to a Guard Column Xbridge-C18 column (4.6 × 200 mm, 5 µm particle size), both purchased from Waters Corporation (Milford, MA, USA).

The analysis was performed using the mobile phase H_2O (pH 4.88):methanol, which was adapted from Calinescu *et al*. (2012), and the following gradient program with respective mobile phase ratios (v/v): 1st ramp from 0 to 8 min with 80:20 to 50:50 (v/v); 1st stationary step from 8 to 11 min with 50:50 (v/v); 2nd ramp from 11 to 12 min with 50:50 to 80:20 (v/v); 2nd stationary step from 12 to 15 min with 80:20 (v/v). The injection volume was 20 µL, the flow rate was set at 0.8 mL/min, the column was maintained at room temperature and detection was done at 244 nm.

3. RESULTS AND DISCUSSION

3.1. Identification of isolates that degrade paracetamol

In order to study which bacteria, within the aerobic community previously studied for drug´s biodegradation (Palma *et al*. 2018), displayed the ability for paracetamol degradation separately from that mixed culture, bacteria were isolated, and assays were performed. Glucose was used as positive control since it is an easily available and often the preferred carbon source in the most part of the documented cases, as for example in the classical example of *Escherichia coli* glucose–lactose diauxic growth (Inada *et al.* 1996; Brückner and Titgemeyer, 2002; Moat *et al.* 2002). Once optimal growth conditions were previously checked, the isolates were incubated at 28 °C, thus corresponding to the optimal environmental conditions for biological treatments. The incubation occurred under dark conditions in order to avoid paracetamol photodegradation.

The bacterial isolates with ability to grow in the presence of 500 mg/L of paracetamol were selected by colony morphotypes, a common procedure in microbial ecology, often used to select clones from complex environments for diversity measurements and/or isolation of the dominant species within cultivable communities (Fredrickson *et al*. 1991; Serratone *et al*. 1995; Atlas, 1984; Jimenez, 1990; Lebaron *et al*. 1998). Colony morphotypes are also used to isolate different species for

physiological and genetic purposes (Martinez *et al*. 1996; Jones *et al.* 1991; Kobori *et al.* 1984; Lebaron *et al*. 1998).

The growth of bacteria at a 10^{-2} dilution in plates with 500 mg/L paracetamol was high, with a colony count from $6.52x10^5$ to $1.22x10^6$ colony forming units (CFU)/mL, after 48 h incubation at 28 ºC in the dark. The positive control at this dilution demonstrated a higher bacterial growth in plaque (>$1.06x10^6$ CFU/mL), after 48 h incubation at 28 ºC in the dark.

From this experiment it was possible to successfully select twelve bacteria with ability to grow in the presence of paracetamol as sole carbon source. The obtained isolates were labeled as 1, 2, 3, 4 until 12. However, only four of the selected isolates have maintained the capacity to grow at 500 mg/L of drug for more than 3 generations, these were the isolates 1, 3, 4 and 9. The study was then focused on these bacteria, whose growth is represented by the appearance of colonies in solid MSM cultures spiked with 500 mg/L of paracetamol (**Figure 4.1**).

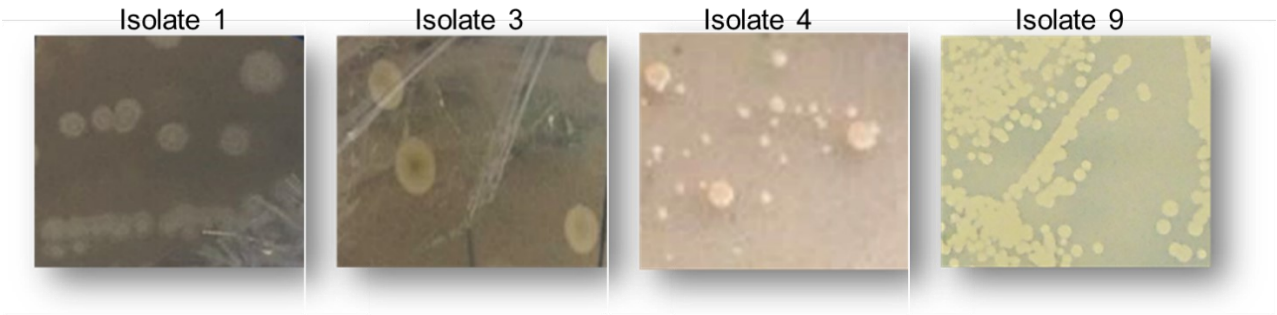

Figure 4.1 Colonies of bacterial isolates 1, 3, 4 and 9 in solid MSM cultures spiked with 500 mg/L of paracetamol

The loss of resistance and ability to grow using paracetamol as carbon source by some of the obtained isolates could be due to the depletion of nutrients and subsequent accumulation of metabolic waste products and other toxic materials in the media that promote the bacterium to move on the death phase. Also, during this process, the bacterium completely loses its ability to reproduce. However, although some bacteria die due to unfavorable conditions some organisms can resist and survive in the environment to this condition by producing endospores (Shimizu, 2013; Robador *et al*. 2018).

The morphological and biochemical characteristics of these four bacterial isolates are described in **Figure 4.1** and **Table 4.1**.

Table 4.1 Morphological and biochemical characteristics of the bacterial isolates which have maintained the ability to use paracetamol as unique carbon source

	Characteristics			
Parameter	**Isolate 1**	**Isolate 3**	**Isolate 4**	**Isolate 9**
Colony morphology	Generally large colonies with a dull or frost-glass surface and undulate margin. A central elliptical endospore is observed	Irregular/ Wrinkled	Slightly irregular/ Smooth	Regular/ Smooth. Pinpoint to small
Colony color	White	Creamy	White	White
Gram reaction	Positive	Positive	Positive	Positive
Morphology	Rod/Non-Motile/Spore-forming/Single, in pairs or chains	Rod/Motile/Endospores	Rod club-shaped/Non-motile/Non-sporing/chains	Cocci/Non-motile/Non-sporing/ grouped in pairs or short chains
Respiration	Facultative anaerobic	Aerobic/ Facultative anaerobic	Strictly aerobic	Facultative anaerobic
Oxidase	Positive	Negative	Positive	Negative
Catalase	Positive	Positive	Positive	Negative

The morphological and biochemical characteristics obtained for each studied isolate are in accordance with those reported in the literature for the bacteria posteriorly identified (Schleifer and Kilpper-Balz, 1984; Liu *et al*. 2017; Logan, 2010; Shin *et al*. 2011; Jarival *et al*. 2015; Rosenberg *et al*. 2016).

The nearly complete 16S rRNA gene sequences of the four strains were determined (1484 bp). The sequence reads obtained by Sanger sequencing were treated using the BIOedit Sequence Alignment Editor and the generated contig-0 sequences (**Table S3; Annex 2**) were analyzed through a BLAST search of Gene Bank database of NCBI and a maximum likelihood phylogenetic tree based on the 16S rRNA gene was constructed (**Figure 4.2**).

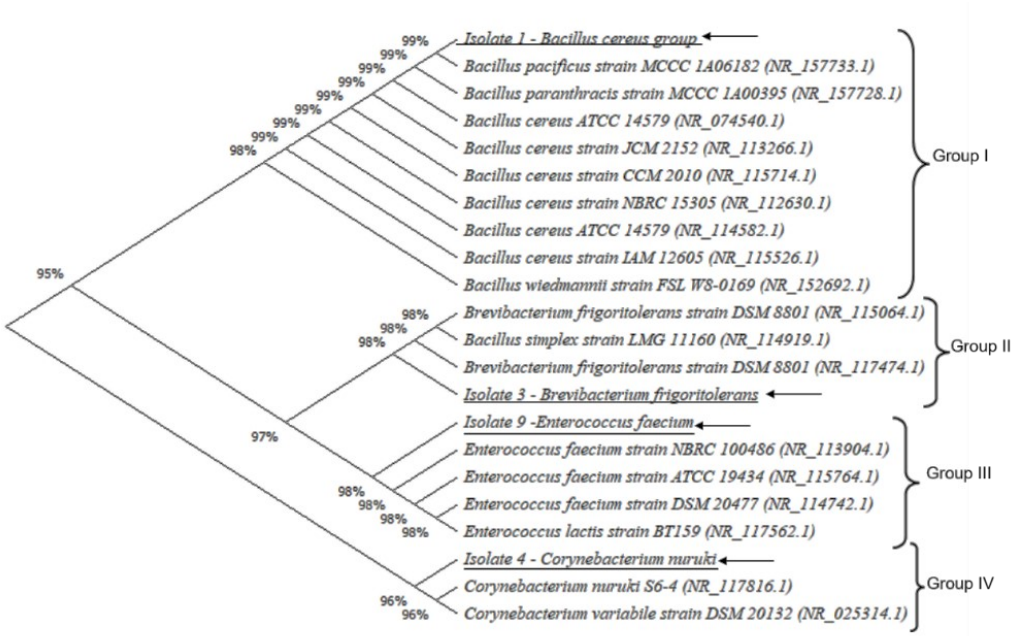

Figure 4.2 Maximum likelihood phylogenetic tree analysis based on the 16S rRNA gene sequences comparison of the four bacterial isolates (*Bacillus cereus* group; *[B.] frigoritoleran*; *E. faecium* and *C. nuruki*) from an aerobic sludge, with ability to grow in the presence of paracetamol as unique carbon source. Node numbers represent bootstrap values (percentage of 500 replications)

Thus, based on the analysis of 16S rRNA sequences, the isolate 1 was identified as *Bacillus pacificus* (type strain MCCC 1A06182) (GenBank accession NR_157733.1) sharing 99.68% of similarity with *Bacillus paranthracis* (type strain MCCC 1A00395) (GenBank accession NR_157728.1) and with several *Bacillus cereus* strains (GenBank accessions which are described in **Figure 4.2** and **Table S4**; **Annex 2**). This isolate showed to be assigned to novel species of *Bacillus cereus* group (Liu *et al*. 2017; Miller *et al*. 2016), sharing similarity with the known species of this group *Bacillus paranthracis sp. nov.*, *Bacillus pacificus sp. nov.*, *Bacillus cereus* and *Bacillus wiedmannii sp. nov.* (**Figure 4.2**) and less than 95% similarity with other species of the genus *Bacillus*. Their chemotaxonomic features also corresponded to those of *Bacillus cereus* group, however morphological and biochemical profile fit more specifically with those displayed by *Bacillus pacificus* and *Bacillus paranthracis*.

The 16S rRNA gene sequence phylogeny (**Figure 4.2, group I**) supports the close relatedness of *Bacillus pacificus sp.* nov. with other members of the *Bacillus cereus* group, as indicated by the ≥99.68% sequence similarity and high bootstrap support values (≥98%). It is known that *Bacillus cereus* group species cannot be delineated based only on 16S rRNA gene sequences (Liu *et al*. 2015), being necessary, for example, to perform a *rpoB* gene analysis to allow for a more discriminatory result (Miller *et al*. 2016). However, the focus of the present work is the search for bacteria which can be used in WWTP paracetamol's degradation processes. Then, as these bacteria can present high toxicity, they are herein presented in order to better understand the drug biodegradation mechanisms.

Therefore, due to the uncertainty of the exact specie, the isolate 1 is, herein, named as *Bacillus cereus* group.

The bacterial isolate 3 presented a very high similarity of 16S rRNA sequences (99.79%) with a strain identified as *[Brevibacterium] frigoritolerans* strain DSM 8801 (GenBank accession NR_117474.1) (**Table S4; Annex 2**) and 99.65% with two *Bacillus simplex* strains LMG 11160 and NBRC 15720 = DSM 1321 (GenBank accession NR_114919.1 and NR_112726.1, respectively). According to the 16S rRNA gene sequence phylogeny it is possible to see a high degree of confidence between *[Brevibacterium] frigoritolerans* strain DSM 8801 and the two *Bacillus simplex* strains (98% bootstrap values) showing that they are closely related (**Figure 4.2, group II**). This specific bacterial strain has a controversial classification. Gelsomino *et al*. (2004) referred *B. frigoritolerans* as a misclassified *Brevibacterium* specie, which do not belong to *Brevibacterium* but which seems to belong to the genus *Bacillus* (Gelsomino *et al*. 2004). Also, according to Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, *[Brevibacterium] frigoritolerans*'s current taxonomic position is incorrect (Beesley *et al*. 2010). Beesley *et al*. (2010) reported that *Brevibacterium frigoritolerans* DSM 8801^{T}'s biochemical profile (**Table 4.1**) was mostly consistent with those of *Bacillus* sp. not within the *B. cereus* group, including the ability to sporulate, thereby providing evidence that supports DSMZ's claim that this strain is actually a misidentified *Bacillus sp.*. According to NCBI taxonomy database information about this strain (NCBI:txid450367) is to be noted that the square brackets ([]) around a *genus* indicates that the name awaits appropriate action by the research community to be transferred to another genus.

The isolates 4 and 9 were identified with high percentage of similarity (>99%) as *Corynebacterium nuruki* S6-4 (GenBank accession NR_117816.1) and *Enterococcus faecium strain NBRC 100486* (GenBank accession NR_113904.1), respectively by NCBI BLAST database (**Figure 4.2, Group III** and **IV** and **Table S4**; **Annex 2**).

The bacteria identified in the present work were already described as having an important role in biodegradation processes due to their characteristics and adaptability. Both "*Bacillus cereus* group" and *[Brevibacterium] frigoritolerans* are members of the genus *Bacillus,* which can produce thick-walled endospores that are resistant to heat, radiation and chemical disinfection (Logan, 2005; Thakur, 2015). For example, several studies reported that *Bacillus cereus* showed more tolerance to a high level of hydrocarbons in soil due to their resistant endospore (Annweiller *et al*. 2000).
Also, members of *Actinomycetes* (Singh, 2017), mainly *Corynebacterium* genera are capable to adhere to and to produce biofilm on hydrophilic glass surfaces (Souza *et al*. 2015).

As already mentioned, the bacterial isolates were obtained from sludge, collected in Faro Northwest WWTP's oxidation ditch, that was previously used in paracetamol biodegradation assays performed in aerobic conditions (Palma *et al*. 2018). In that previous work several bacteria were identified, as members of the bacterial community: a strain from genus *Pseudomonas* with the highest final relative abundance (21.2%) and also the genera *Flavobacterium*, *Dokdonella* and *Methylophilus* were in evidence, with relative abundances of 6.91%, 3.80 and 3.83% after incubation, respectively (Palma *et al*. 2018). Although one of the objectives of this work was to verify which of the bacteria present in that consortium were able to degrade paracetamol alone, none of the 25 most abundant bacteria were identified among the obtained isolates. Two possible explanations for this event could be:

a) the fact that the sludge was stored at 4 ºC for 6 months, might have cause a decrease in the original population responsible for paracetamol biodegradation by the community (Palma *et al*. 2018) or/and
b) the most abundant bacteria which belong to the community may not be able to grow isolated from the other bacteria in the presence of the drug.

As these bacterial isolates were resistant to paracetamol and presented the capacity to grow in the presence of this drug as unique carbon source, it can be inferred that the bacteria likely degraded it. Thus, liquid assays were performed using the four selected isolates, in order to test drug degradation and the formation of paracetamol secondary metabolites along the process.

3.2. Paracetamol degradation by bacterial isolates

3.2.1. Paracetamol degradation by bacterial isolates in liquid cultures

In order to study the degradation of paracetamol by the previous bacterial isolates, assays in MSM liquid medium spiked with 200 mg/L and 500 mg/L of paracetamol were performed (**Figure 4.3**).

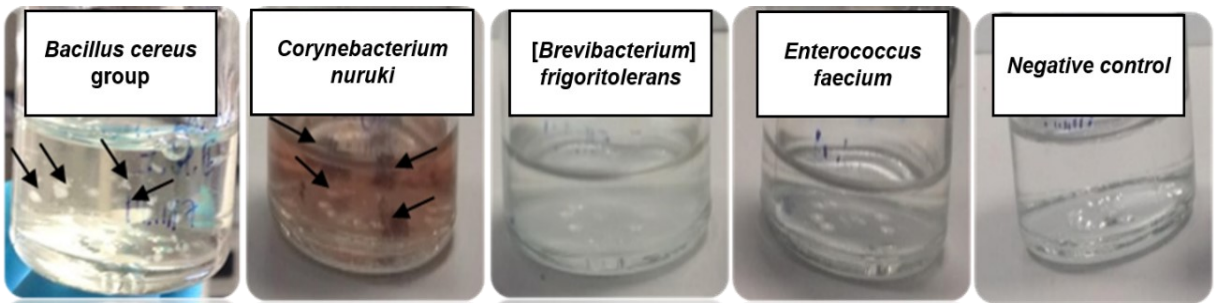

Figure 4.3 Representation of the culture characteristics displayed by each isolate ("*Bacillus cereus* group"*, Corynebacterium nuruki, [Brevibacterium] frigoritolerans* and *Enterococcus faecium)* in liquid MSM spiked with 200 mg/L paracetamol after 144 h incubation, in dark at 28ºC. The same characteristics were displayed with 500 mg/L of paracetamol with exception of the negative control that developed a slightly yellowish color. Arrows pointing to bacterial clumps

The turbidity of the cultures was measured at 600 nm in order to analyze the bacterial growth. For "*Bacillus cereus* group" species and *Corynebacterium nuruki* there was formation of bacterial aggregates and glass adherent biofilm (**Figure 4.3**) making it difficult to correctly measure the optical density for both positive control and in the presence of 200 and 500 mg/L of paracetamol. The maximum growth obtained at 600 nm for *[Brevibacterium] frigoritolerans* was 0.60 ± 0.06 and 0.42 ± 0.05 and for *Enterococcus faecium* was 0.48 ± 0.06 and 0.26 ± 0.04, in MSM spiked with 200 and 500 mg/L of paracetamol, respectively, during the assay of 144 h at 28 ºC in dark. In cultures of *[Brevibacterium] frigoritolerans* and *Enterococcus faecium* without drug (positive control) the growth was of 0.95 ± 0.09 and 0.76 ± 0.08, respectively, after 144 h incubation.

This bacterial flocking/clumping can occur as a natural way for species to grow, but this can also be a mechanism to avoid the media toxicity (Crosby *et al.* 2016) or, a mechanism of protection of some anaerobic species that allow them to grow in aerobic conditions (usually facultative species, not strict anaerobes, as is the case of the *Bacillus cereus* group members).

In addition, since there is no information about *Corynebacterium nuruki,* its behavior can be inferred by some other studied *Corynebacterium* species, such as *Corynebacterium jeikeium*, *Corynebacterium macginleyi* and C. *diphtheriae* which were found to adhere to available biotic and abiotic surfaces and/or to form biofilms (Wang *et al*. 2001; Kwaszewska *et al*. 2006; Suzuki *et al*. 2007). For example, *Corynebacterium pseudodiphtheriticum* (CPS), a commensal bacterium that colonizes skin and mucosal sites, has become progressively multiresistant, due to its capacity to multiply attached and form microcolonies that accumulate as multilayered cell clusters, a step that involves intercellular adhesion and synthesis of extracellular matrix molecules. Further growth led to the formation of dense bacterial aggregates embedded in the exopolymeric matrix surrounded by voids, typical of mature biofilms (Souza *et al*. 2015). This type of bacterial formation seems to have been the same as occurred with *Corynebacterium nuruki* in the presence of paracetamol.

The obtained results of paracetamol removal, in liquid MSM spiked with 200 mg/L and 500 mg/L of the drug and inoculated with each one of the four bacterial isolates, after 48 h and 144 h at 28 ºC in dark conditions, are presented in the **Figure 4.4.**

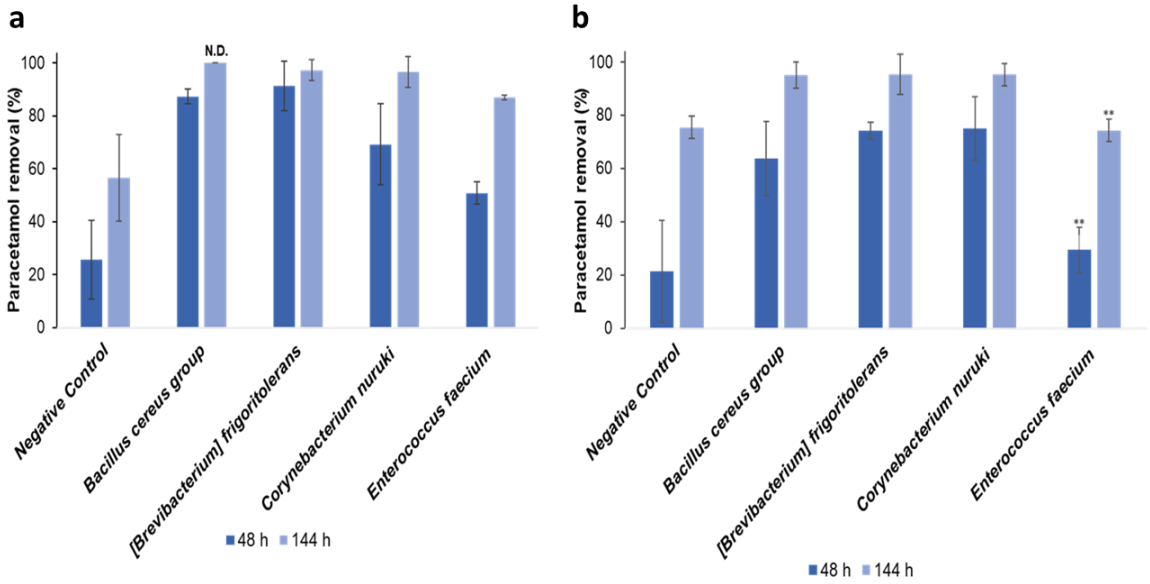

Figure 4.4 Paracetamol removal (%) in MSM liquid medium in the presence of 200 mg/L (a) and 500 mg/L (b) paracetamol inoculated with each one of the four bacterial isolates, after 48 h and 144 h at 28 ºC in dark conditions (n=3; mean ± standard deviation). N.D. = Not Detected, below the limits of detection. ** = No significant difference compared to the negative control ($p>0.05$)

When members of the species "*Bacillus cereus* group"*, [Brevibacterium] frigoritolerans* and *Corynebacterium nuruki* were inoculated in the presence of 200 and 500 mg/L of paracetamol a high percentage of drug removal was achieved.

"Bacillus cereus group"*, [Brevibacterium] frigoritolerans*, *Corynebacterium nuruki* and *Enterococcus faecium* removed about 87 ± 3%, 91 ± 9%, 69 ± 15% and 51 ± 4% of 200 mg/L of paracetamol, respectively, after 48 h. *[Brevibacterium] frigoritolerans, Corynebacterium nuruki* and *Enterococcus faecium* degraded about 97 ± 4%, 97 ± 6% and 86.9 ± 0.8% of 200 mg/L of the drug, respectively, after 144 h incubation (**Figure 4.4a**). On the other hand, a specie member of "*Bacillus cereus* group" degraded below the limits of detection 200 mg/L of paracetamol (**Figure 4.4a**), without giving rise to toxic metabolites, since the peaks corresponding to 4-aminophenol, hydroquinone or benzoquinone were not detected during the chromatographic analyzes, after 144 h incubation (**Figure S1; Annex 2**). On the other hand, cultures spiked with 200 mg/L of paracetamol without bacteria (negative control) presented a drug removal of 26 ± 15% and 56 ± 16% after 48 h and 144 h assay, respectively. Thus, when compared with the negative control "*Bacillus cereus* group" specie*, [Brevibacterium] frigoritolerans, Corynebacterium nuruki* and *Enterococcus faecium* caused a significant decrease ($p<0.05$) in 200 mg/L of paracetamol, after 48 and 144 h incubation (**Figure 4.4a**).

Cultures of "*Bacillus cereus* group"*, [Brevibacterium] frigoritolerans* and *Corynebacterium nuruki* spiked with 500 mg/L of paracetamol displayed 64 ± 14%, 74 ± 3% and 75 ± 12% of drug removal, respectively, after 48 h incubation. In the negative control after 48 h of incubation the removal of the drug was of 21 ± 19%. Thus, the isolates "*Bacillus cereus* group", *[Brevibacterium] frigoritolerans* and *Corynebacterium nuruki* caused a significant decrease ($p<0.05$) at 500 mg/L of the drug, after 48 h assay (**Figure 4.4b**).

For assays in the presence of 500 mg/L paracetamol, after 144 h of incubation, cultures of "*Bacillus cereus* group", *[Brevibacterium] frigoritolerans* and *Corynebacterium nuruki* displayed a degradation of 95 ± 5%, 95 ± 8% and 95 ± 4% of the drug, respectively. Although, the negative control presented a very high removal percentage of 75 ± 4%, the isolates "*Bacillus cereus* group", *[Brevibacterium] frigoritolerans* and *Corynebacterium nuruki* removed significantly ($p<0.05$) 500 mg/L of paracetamol after 144 h, when compared to this control.

On the other hand, for cultures of *Enterococcus faecium* spiked with 500 mg/L of paracetamol the removal of the drug was about 29 ± 8% and 74 ± 4%, respectively, after 48 h and 144 h of incubation (**Figure 4.4b**), revealing no significant differences ($p>0.05$) compared with the negative control performed in the same assay conditions.

In the negative control (without bacteria) the high percentages of paracetamol degradation after 144 h of assay could be due to several conditions and factors that may affect the stability of paracetamol, such as the ones described below.

Paracetamol is known to be unstable under humid conditions and the rate of degradation of paracetamol in aqueous solutions is enhanced by the increase of temperature, exposure to light, changes in solution's pH outside the range of 5-6 (paracetamol breakdown is minimal at a pH close to 6 at 25 ºC), and by contamination with traces of 4-aminophenol (Paracetamol IARC monographs; Dietlin and Fredj, 2000; Chiam *et al.* 2015). Dietlin and Fredj (2000) reported that a paracetamol solution in water at 50 ºC suffered degradation after 2 weeks. They also referred that exposure of paracetamol to moisture can result in hydrolysis with formation of 4-aminophenol, followed by oxidation, with appearance of a pink color, typical of the production of quinonimine. In some aqueous solutions 4-aminophenol was not detected, thus the breakdown of paracetamol first involves an oxidative process followed by hydrolysis. "According to this theory, paracetamol may react with an oxidant present in solution, for example oxygen dissolved in the aqueous layer. This mechanism may involve the production of free radicals resulting in molecular coupling, a fact that may account to produce colored derivatives evolving in color from pink to brown" (Dietlin and Fredj, 2000).

The negative controls were submitted to the same incubation conditions than the assays with bacteria, under agitation at 28 ºC, which favored the oxygenation of cultures. However, the oxygen

that was not used by bacteria became available to react with paracetamol leading to the oxidation and subsequent hydrolysis of the drug, speeding up its degradation. In previous studies, our group found that bacteria had a buffering effect in cultures, maintaining the pH of the solution stable. In the absence of bacteria and with the occurrence of chemical reactions, the pH may suffer significant changes affecting the stability of paracetamol. Khamis and colleagues (2011) also found that the drug undergo hydrolysis to 4-aminophenol when kept standing for some time (7 days) in wastewater sludge (Khamis *et al.* 2011).

Although these specific bacterial isolates have not yet been described in the literature regarding their role in paracetamol biodegradation, for some of them several studies have been conducted in what concerns their biodegrading activity on other organic compounds. As there is a lack of information to compare some of these isolates specifically, examples of biodegradation studies with bacteria of the same *genus* are herein presented.

Results obtained by Ijah and Ukpe (1992) suggested that two *Bacillus* strains (28A and 61B) can play an active role in breaking down petroleum pollutants in soil (Ijah and Ukpe, 1992). Members of *Bacillus spp*. were assumed to effectively degrade pesticides and therefore play an important role in bioremediation of pesticide contaminant sites (Islam *et al*. 2016). *Bacillus clausii T* removed 100% of a mixture of cefuroxime (CFX), cefotaxime (CTX), and cefpirome (CPR) within 8 h (Kong *et al*. 2019). Patowary *et al*. (2016) studied the biodegradative capacity for total petroleum hydrocarbons of 23 bacterial isolates from petroleum-contaminated soils, and subsequently designed 14 artificial consortia based on the five most efficient isolates. The best designed consortium, comprised of two biosurfactant-producing, hydrocarbon-degrading strains of *Bacillus pumilus* and *Bacillus cereus*, showed up to 84% degradation of total petroleum hydrocarbons after five weeks (Patowary *et al*. 2016; Dvorak *et al*. 2017). As member of *Bacillus cereus* group, *Bacillus thuringiensis* B1(2015b) was reported by Marchlewicz *et al*. (2017) as degrading ibuprofen and naproxen with high efficiency in the presence of glucose, as an additional carbon source. Also, Ferreira *et al*. (2016) reported phenantherene degradation by *Bacillus thuringiensis*.

Jariyal *et al*. (2015) shown that *Brevibacterium frigoritolerans* strain IMBL 2.1 completely degraded phorate, an organophosphorus insecticide, within 7 days. This bacterium was considered the most effective of phorate degraders and hence was selected for further studies aiming to optimize their bioremediation potential in agricultural soils (Jariyal *et al*. 2015).

In what concerns, *Corynebacterium* genus, it is described in the literature as having the ability to degrade phenol compounds and petroleum oil (Ho *et al*. 2009, Aouad and Abbouni, 2012). On the other hand, *Actinobacteria* has been recently identified in WWTPs as an accumulator, more specifically as polyphosphate-accumulating organisms (PAO), being responsible to remove phosphorous from the wastewaters. Thus, accumulation can be an alternative to pollutants

mineralization. These bacteria have been observed in some enhanced biological phosphorus removal (EBPR) plants in Denmark (where they were discovered), but their wider distribution is unknown (Seviour *et al*. 2003).

Although the obtained results were not entirely favorable regarding the paracetamol removal by *Enterococcus* genus, according to the literature, *Enterococcus* can degrade azo dyes in wastewater (Lim *et al*. 2013), while another study indicates that these bacteria can degrade persistent organic compounds, such as decabromodiphenyl, under aerobic conditions (Tang *et al*. 2016). Also, Chen *et al*. (2018) reported for the first time the degradation ability of trimethylamine (TMA) by gut bacteria of human namely 3 *Enterococcus faecalis* isolates (*E. faecalis* Ef1, *E. faecalis* Ef2 and *E. faecalis* Ef3), which could enable TMA degradation anaerobically. The results showed that these strains attenuated TMAO levels after 14 days of administration (Chen *et al*. 2018).

As mentioned, paracetamol biodegradation by a bacterial consortium was already tested by our group (Palma *et al*. 2018), thus the main objective of this work was to emphasize the importance of the isolates in the paracetamol degradation. However, a preliminary test was performed by pooling the three best isolates ("*Bacillus cereus* group", *[Brevibacterium] frigoritolerans* and *Corynebacterium nuruki*) in the presence of 200 mg/L of paracetamol. The main aim of this assay was to verify which improvements could be obtained by adding the best bacterial isolates together, thereby designing an artificial consortium with a high paracetamol biodegradation efficiency. In this experiment the drug was totally degraded, after 48 h with apparent mineralization, thus evidencing an improvement in comparison with the results previously obtained for each isolate individually in the same conditions (**Figure 4a**). The obtained results seem to be promising since that none of the isolates, with exception of the *"Bacillus cereus* group", showed the ability to degrade paracetamol below LOD, during the "individual" assay, and despite the good results, even the *"Bacillus cereus* group" only accomplished paracetamol's degradation below the limits of detection, after 144 h of incubation. However, further studies should be done in order to investigate the behavior (*e.g.* resistance, stability and biodegradation capacity) of this consortium over generations.

3.3. Possible degradation pathway of paracetamol by the bacterial isolates

Paracetamol consists of a benzene ring core substituted by one hydroxyl group and the nitrogen atom of an amide group in the para (1, 4) pattern (Bales *et al*. 1985). As part of the class of drugs known as "aniline analgesics", paracetamol is the only drug of such class still in use today.

As already referred, biodegradation of pharmaceutical compounds is considered as an environmentally friendly and low-cost option and it has been demonstrated to have the potential to eliminate drugs by degrading them into innocuous end products such as CO_2 and H_2O (Hasan *et al*. 2011; Chen *et al*. 2010).

Microorganisms have established effective strategies involving specialized enzyme systems and metabolic pathways to access paracetamol as a carbon and energy source. For example, Ahmed *et al*. (1999) reported that *Pseudomonas* sp. was capable to use aromatic compounds as carbon energy source, under aerobic conditions. Zhang *et al*. (2012) isolated three bacterial strains which were able to use paracetamol as the unique carbon, nitrogen, and energy source from a paracetamol degrading aerobic community, that were assigned to species of the genera *Stenotrophomonas* and *Pseudomonas* (Wu *et al.* 2012; Zhang *et al*. 2012). They reported that during the biodegradation of paracetamol and after 2 h of incubation (MSM, initial concentration 500 mg/L, 28 °C, pH 7.2), a total of ten intermediates were identified including 4-aminophenol, hydroquinone and hexenoic acid.

A chromatogram with representative peaks corresponding to paracetamol and its metabolic products: 4-aminophenol (4.90 min), hydroquinone (5.52 min), paracetamol (6.63 min), benzoquinone (7.27 min), 2-hexenoic acid (12.30 min) and 4-nitrophenol (13.35) is presented in **Figure S1**; **Annex 2**.

In the present study, for cultures spiked with 200 mg/L of paracetamol incubated with "*Bacillus cereus* group", *Corynebacterium nuruki* and *Enterococcus faecium* peaks that can match to hydroquinone were detected, while for the isolate *[Brevibacterium] frigoritolerans* peaks matching with 4-aminophenol (4.57 min) and hexenoic acid (12.53 min) appeared after an incubation period of 48 h (**Figure S2a; Annex 2**).

A specie member of "*Bacillus cereus* group" degraded the drug to values below the limits of detection with evidence of mineralization since no peak was detected, whereas in cultures of isolate *[Brevibacterium] frigoritolerans* 2.8% of paracetamol remained to degrade, nevertheless no other important compounds were detected after 144 h incubation. In the case of *Corynebacterium nuruki,* 3.4% of paracetamol and residual concentrations of hydroquinone were detected, whereas for *Enterococcus faecium* a higher percentage of paracetamol (13.1%) remained to degrade (**Figure S2b; Annex 2**) after 144 h.

On the other hand, for cultures spiked with 500 mg/L of paracetamol incubated with "*Bacillus cereus* group", a peak that corresponds to hydroquinone was detected (5.38 min) after 48 h incubation, whereas peaks corresponding to 4-aminophenol (4.88 min), 4-nitrophenol (13.18 min) and hexenoic acid (12.50 min) were identified after 144 h (**Figure S3a, b; Annex 2**). In *[Brevibacterium] frigoritolerans* cultures at 500 mg/L of paracetamol it was possible to detect "unknown" peaks, since they did not have any correspondence with the studied compounds and a peak at 12.97 min that could match to hexenoic acid also appeared, after 48 h incubation. Moreover, after 144 h incubation, hydroquinone (5.52 min) and hexenoic acid (12.30 min) were detected. For both *Corynebacterium nuruki* and *Enterococcus faecium* a peak corresponding to hexenoic acid

(12.37 min) was found after 48 h incubation and a peak likely corresponding to benzoquinone (7.45 min) was identified after 144 h (**Figure S3a**; **Annex 2**). As shown in **Figure 4.3**, the culture with *Corynebacterium nuruki* presented a brown color, that may be correlated with the secondary metabolite 4-aminophenol, the main responsible for this medium coloration. This is in accordance with the chromatogram (**Figure S3**; **Annex 2**), where it was detected a residual concentration of 4-aminofenol at 5.68 min, after 48 and 144 h of assay.

Looking to the obtained results it may be predicted that the mechanism of paracetamol degradation by these isolates occurs in the same away as already described in other studies.

Thus, the present study corroborates the literature since paracetamol conversion by bacteria was proposed to proceed via 4-aminophenol to hydroquinone, which is described to be the major route in its biodegradation (Wu *et al.* 2012). Paracetamol ($C_8H_9NO_2$) is initially catalyzed by an amidohydrolase occurring the cleavage of the bond between nitrogen and carbon from the carbonyl group with subsequent release of acetate to yield 4-aminophenol (C_6H_7NO), in which the amino group (nitrogen elimination) is then replaced by a hydroxyl group (hydroxylation) to form hydroquinone or 1,4-benzenediol ($C_6H_6O_2$), subsequent to ring fission (Wu *et al.* 2012; Zhang *et al*. 2012; Li *et al*. 2014). Zhang *et al*. (2012) reported that in the second instance, the initial hydroxylation of paracetamol could potentially be catalyzed by a hydrolytic enzyme to produce hydroquinone ($C_6H_6O_2$) with release of acetamide, which could be further converted into oxamic acid ($C_2H_3NO_3$). Hydroquinone ($C_6H_6O_2$) was observed as a transient metabolite, as it could be further oxidized to 1,4-Benzoquinone ($C_6H_4O_2$) that is more stable and thought to be a critical toxic metabolite of benzene exerting genotoxic and mutagenic effects (Smith, 1996; Snyder and Hedli, 1996). Also, the generation of N-acetyl-p-benzoquinone imine (NAPQI) ($C_8H_7NO_2$), a highly reactive and toxic metabolite of paracetamol which is associated with hepatic necrosis (Tsikas *et al*. 2011), was found as another oxidation product from paracetamol. De Gusseme *et al*. (2011) also reported that hydroquinone was a temporary transformation product of paracetamol degradation by two bacterial strains, *D. tsuruhatensis* and *P. aeruginosa*. De Gusseme *et al*. (2011) described that the methylation of hydroquinone may further result in the mono and di O-methylated intermediates 4-methoxyphenol ($C_7H_8O_2$) and 1,4-dimethoxybenzene ($C_8H_{10}O_2$).

Li *et al.* (2014) identified a total of 8 intermediates, including 3-hydroxyacetaminophen, hydroquinone, 1,4-benzoquinone, N-acetyl-p-benzoquinone imine, p-acetanisidide, 4-methoxyphenol, 2-hexenoic acid, and 1,4-dimethoxybenzene. This group proposed that the relatively high concentration of 2-hexenoic acid ($C_6H_{10}O_2$) at 13.58 min, in the samples suggested the cleavage of the benzene ring and may have contributed to the total paracetamol mineralization.

In the case of paracetamol biodegradation by "*Bacillus cereus* group", *Corynebacterium nuruki*, *[Brevibacterium] frigoritolerans and Enterococcus faecium*, the drug was likely also metabolized

via an amidohydrolase reaction, with a bond cleavage between nitrogen and carbon from the carbonyl group producing the 4-aminophenol from which nitrogen elimination followed by hydroxylation would lead to the formation of hydroquinone and further cleavage of the benzene ring likely forming 2-hexenoic acid leading to subsequent mineralization.

To the best of our knowledge, our study is the first to demonstrate the paracetamol-degrading ability by a specie member of "*Bacillus cereus* group", *[Brevibacterium] frigoritolerans, Corynebacterium nuruki and Enterococcus faecium*.

4. CONCLUSIONS

In the present study bacteria with ability to degrade paracetamol were isolated from aerobic sludge from an oxidation ditch's WWTP and identified utilizing the 16 S rRNA bacterial gene. This study shows for the first time, to our knowledge, that members of *Bacillus cereus* group assigned to *Bacillus pacificus* strain MCCC 1A06182 or *Bacillus paranthracis* strain MCCC 1A00395, *[Brevibacterium] frigoritolerans*, *Corynebacterium nuruki and Enterococcus faecium* were able to biodegrade paracetamol. Experiments using these isolates suggested that a "*Bacillus cereus* group" species, *[Brevibacterium] frigoritolerans*, *Corynebacterium nuruki* were able to use 200 and 500 mg/L of paracetamol as unique carbon source. A specie from *Bacillus cereus* group degraded below the limits of detection 200 mg/L of paracetamol and removed about 95 ± 5% of 500 mg/L of the drug, after 144 h of incubation. In both concentrations the degradation occurred apparently without giving rise to toxic secondary metabolites, after 144 h of incubation at 28 ºC. *Enterococcus faecium* was able to degrade 200 mg/L of paracetamol but did not cause a significant decrease ($p>0.05$) of the drug at 500 mg/L.

4-aminophenol, hydroquinone, that possess known biological activity, and 2-hexenoic acid were detected as metabolic degradation intermediates of paracetamol by *[Brevibacterium] frigoritolerans* and *Corynebacterium nuruki*, during 144 h of experiment.

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CHAPTER 5

Biodegradation of fluoxetine in anaerobic systems and associated microbial community dynamics

A modified version of this chapter would be published as:

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ABSTRACT

Fluoxetine (FLX), an antidepressant used worldwide is considered an emerging pollutant. The present work intends to find an anaerobic bacterial community of sulphate-reducing bacteria (SRB) isolated from a lagoon system wastewater treatment plant, with capacity to biodegrade and use FLX as sole carbon source, since the current literature suggests that this drug is poorly biodegraded being mainly removed by adsorption to sediments, where it persists. Biodegradation studies were performed in modified Postgate B a specific medium for SRB growth in the presence of lactate plus 20 and 50 mg/L of FLX or only with the drug as carbon source. A mineral salt medium was also tested as well as the possibility of FLX adsorption to sludge. The identification of the bacterial populations was performed through metagenomic analysis of the 16S rRNA gene sequences. In the presence of FLX as sole carbon source, the results showed that the consortium removed 20 mg/L of FLX below LOD and 66 ± 9% of 50 mg/L FLX, after 31 days and from their degradation resulted 2,3-dimethyl-5-(trifluoromethyl)benzene-1,4-diol and norfluoxetine as secondary metabolites, respectively. The initial bacterial population was mainly constituted by *Desulfomicrobium* and *Desulfovibrio*, whereas during the experiments using FLX as unique carbon source a clear shift occurred with the increase of *vadinBC27* wastewater-sludge group, *Macellibacteroidetes, Dethiosulfovibrio, Bacteroides, Tolumonas, Sulfuricurvum,* f_*Enterobacteriaceae*_OTU_18. These bacteria are assumed for the first time as FLX biodegraders. Although the main mechanism of FLX removal described in literature is by adsorption, in the results herein presented biodegradation appears to play the main role in the removal of the FLX.

Keywords: Fluoxetine, Secondary metabolites, Sulphate-Reducing Bacterial community, Biodegradation

1. INTRODUCTION

Emerging pollutants (EPs) are defined as naturally or synthetic occurring chemicals which are not normally present in the environment. EPs are currently not included in international routine monitoring programmes and their fate, behaviour and ecotoxicological effects are often not well understood (Escher *et al*. 2011; Vulliet and Cren-Olivé, 2011; Geissen *et al*. 2015).

Fluoxetine (FLX) or (R,S)-N-methyl-3-phenyl-3-(4-(trifluoromethyl)phenoxy)propan-1-amine (CAS No: [54910-89-3]) is included on that wide used pharmaceutical drugs considered as EPs. This pharmaceutical drug is a diphenhydramine derivative and a chiral fluorinated pharmaceutical constituted by (R)- and (S)- enantiomers, where (R)-FLX was also found to be more potent than the S-isomers (Stevens and Wrighton 1993). FLX selective serotonin reuptake inhibitor (SSRI) is indicated mainly for treatment of depression, panic, anxiety, or obsessive-compulsive symptoms and is one of the most prescribed drugs in the world. FLX (**Figure 5.1a**) is mainly metabolized by humans to Norfluoxetine (NFLX) (**Figure 5.1b**) by CYP450N-demethylation and to several other metabolites such as FLX glucuronide, NFLX glucuronide. The metabolism of FLX and NFLX is through O-dealkylation releasing *p*-trifluoromethylphenol, which is further metabolized to hippuric acid ($C_9H_9NO_3$) (Benfield *et al*. 1986; Andrés-Costa *et al*. 2017).

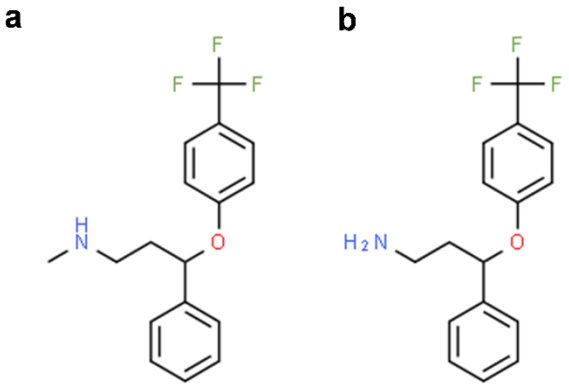

Figure 5.1 Molecular structure of FLX (a) and NFLX (b) (ChemSpider database 2019)

The main path of drug excretion is via urine (80%) with 11.6% of it reported to be excreted as FLX unchanged, 7.4% as FLX glucuronide, 6.8% as NFLX, 8.2% as NFLX glucuronide, >20% hippuric acid, 46% other compound (Benfield *et al*. 1986; Andrés-Costa *et al*. 2017) and approximately 15% excreted in the faeces (Andrés-Costa *et al*. 2017).

Environmental contamination with this drug and its active metabolite NFLX has been detected in sewage effluents and in surface waters (Metcalfe *et al*. 2010; Petrie *et al.* 2014).

The total concentrations of the respective enantiomers of FLX and NFLX were detected between 3.5 to 16 ng/L in raw wastewater and between 1.2 to 15 ng/L in treated wastewater, the same

concentration ranges were found in other studies performed in Europe and in North America. Interestingly, the Predicted Environmental Concentrations (PEC) of (R,S)-FLX in raw wastewater in Spain have been determined to be in a range from 80 to 200 ng/L (Barclay *et al*. 2012). FLX and NFLX were found in United Kingdom's wastewater influents in the range of 4.9 - 175.9 ng/L and 3.4 - 118 ng/L, respectively and in the effluents were detected ranging 5.6 - 44.9 ng/L FLX and 1.1 - 20.2 ng/L NFLX (Baker and Kasprzyk-Hordern, 2013). In Portugal, FLX was only detected in the influent wastewaters, with a frequency of 5%, in a mean concentration of 127.97 ng/L (14.6 mg/day/1000 inhab.) (Silva *et al*. 2014).

Recent research studies have shown that most part of drugs, including FLX and NFLX, enter in the water resources by untreated municipal wastewater, being detected in these from ng/L to μg/L (Andrés-Costa *et al*. 2017). Benotti *et al*. (2009) found that United States tap water was contaminated with 0.64 ng/L FLX and 0.77 ng/L NFLX. Furthermore, fish collected near the municipal wastewater discharges were contaminated with these compounds (Andrés-Costa *et al*. 2017). Studies performed by Brooks *et al*. (2003) demonstrated that FLX became hazardous to the aquatic environment at concentrations at least with an order of magnitude higher than that reported in municipal effluents that ranged from 0.3 to 1.6 nM. Contrary to pesticides the drug concentrations detected in the environment are not acutely toxic to aquatic organisms (Brooks *et al*. 2003). Exposure of non-target organisms to drugs at nanomolar or even picomolar concentration can lead to chronic responses that remain to be identified (Fong *et al.* 1998). The municipal effluents with an upstream dilution present a benefit to the aquatic environments reducing the maximal risk of contamination (Fong *et al.* 1998; Brooks *et al*. 2003). Both FLX and NFLX remain biochemically active in the aquatic environments and may cause remarkable harmful effects on the morphology, physiology, and behaviour of different organisms (Fong *et al.* 1998; Brooks *et al*. 2003; Andrés-Costa *et al*. 2017). These compounds frequently act by mimicking the neurotransmitter serotonin, whose function is to regulate several physiological systems in fish, molluscs, and protozoans, causing significant effects even at residual concentrations, on these and other aquatic organisms (Silva *et al*. 2012). Some of the hazardous effects described include biological activity changes of aquatic species, behavioural disturbance, reproduction declination, abnormalities in embryo development, retard in physiological development and sexual maturation (Santos *et al*. 2010; Silva *et al*. 2012; Silva *et al*. 2014; Weinberger and Klaper, 2014). Experiments with fish exposed to FLX revealed anatomical changes, additionally, FLX at 28 ng/L induced vitellogenin in male fish, a typical consequence for estrogenic endocrine disruption (Silva *et al*. 2014). Shi *et al*. (2019) found that FLX may be immunotoxic to *Tegillarca granosa* clams through an overarching mechanism by reducing cell viability, modifying Ca^{2+} homeostasis, disturbing neurotransmitter signal transduction, which subsequently generates a stressful condition.

FLX's behaviour was investigated in laboratory experiments, through studies with several aqueous solutions, water/sediment systems, and activated sludge-amended medium. Kwon and Armbrust (2006) reported that during 30 days of experiment, FLX did not suffer hydrolysis and photolysis, remaining stable in all aqueous solutions except in synthetic humic water (pH 7), where an increase of approximately 13-fold in the drug's degradation was detected in comparison with buffered solutions at the same pH. They also demonstrated that the fast decay of FLX from the aqueous phase in water/sediment systems occurred mainly due to its distribution to sediments. Therefore, the adsorption to sediments may reduce the possible impact of FLX in aquatic environments (Kwon and Armbrust, 2006). Based on results of "ready-biodegradability" studies, it is not expected that FLX suffer a fast biodegradation in wastewater treatment plants. Kwon and Armbrust (2006) detected NFLX, which was produced by demethylation, as the only metabolite formed during the photolysis in a sample of synthetic humic water.

Also, Kinney *et al*. (2006) reported adsorption of FLX to biosolids produced by wastewater treatment plant. Cartwright *et al*. (2010) reported that the removal efficiency of FLX in an activated sludge treatment was over 90%, but it was adsorbed by sludge and not biodegraded. Also, Moreira *et al*. (2015) investigated about FLX adsorption to aerobic microbial granules and proposed the development of an adsorption/desorption mechanism for the removal of this pharmaceutical from wastewater.

Thus, the literature suggests that FLX and NFLX hardly suffer hydrolysis, photolysis, and microbial degradation but they revealed to be quickly removed from surface waters by adsorption to sediments, where they seem to be persistent (Cartwright *et al*. 2010; Kinney *et al*. 2006; Kwon and Armbrust, 2006; Moreira *et al*. 2015; Peake *et al*. 2015).

However, all the effects caused by FLX and NFLX in the environment are still unknown, since laboratory and "field-based" investigations of FLX removal by activated sludge systems or within the receiving environments are scarce (Lam *et al*. 2005; Kwon and Armbrust, 2006; Oakes *et al*. 2010; Peake *et al*. 2015). Yet, given that drug's metabolites can also be recalcitrant, persistent and even more toxic than the parent compound, it is essential to determine their behaviour and destination in biological systems (Mbokou *et al*. 2016; Pereira *et al*. 2016; Santos *et al*. 2010; Peake *et al*. 2015); hence, being necessary to investigate for new, effective, economic and ecological ways to degrade or remove them from wastewater treatments.

Microorganisms, in particular bacteria have already demonstrated to play a fundamental role on the biodegradation of organic compounds (Pieper and Reineke, 2000). The present study focuses on the search for anaerobic bacterial communities, considering the well-known advantages of consortia over pure cultures (Mukred *et al*. 2008), with capacity to biodegrade FLX, since the previous studies mentioned in the literature refer that its removal occurs mainly by adsorption processes.

For that purpose, a sludge collected in an anaerobic lagoon system was enriched to favour the growth of SRB community, which was later used in the FLX biodegradation assays. The experiments were performed in cultures using a modified Postgate B medium in the presence of lactate plus FLX or only with the drug as carbon source. A mineral salt medium (MSM) was also used in order to test FLX as sole carbon source, under anaerobic conditions. The resultant metabolic products were analysed in order to elucidate about FLX biodegradation and the bacterial community evolution during the experiment was evaluated through its identification by metagenomic analysis of the 16S rRNA gene sequences.

2. MATERIALS AND METHODS

2.1. Chemicals

The following reagents were purchased from Panreac (Barcelona, Spain): ammonium chloride (NH_4Cl), ammonium hydroxide (NH_4OH), ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$)), calcium chloride ($CaCl_2$), cobalt(II) chloride hexahydrate ($CoCl_2.6H_2O$), manganese(II) sulfate ($MnSO_4$), magnesium chloride hexahydrate ($MgCl_2.6H_2O$), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), sodium chloride (NaCl), sodium lactate ($C_3H_5NaO_3$), sodium sulfate (Na_2SO_4), zinc sulfate ($ZnSO_4$). Ascorbic acid, ethylenediamine tetraacetic acid (EDTA), potassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) both AnalaR NORMAPUR® were obtained from VWR Prolabo Chemicals (Leuven, Belgium). Yeast extract was acquired from HiMedia Laboratories (Mumbai, India) and thioglycolic acid was purchased from Merck (Darmstadt, Germany). All the solutions of fluoxetine hydrochloride (99% purity), norfluoxetine hydrochloride (99% purity) were purchased from Sigma-Aldrich (Deisenhofer, Germany). Acetonitrile (ACN) and methanol, both HPLC grade, and phosphoric acid (85% purity) were supplied by VWR Prolabo Chemicals (Fontenay-sous-Bois, France).

2.2. Inocula sources and preparation

A SRB consortium was enriched from an anaerobic sludge collected from Faro East WWTP's lagoon system (anaerobic pond) in Postgate B medium and was used as inoculum to test the ability of that community in FLX degradation under anaerobic conditions.

The enriched community of SRB were grown in a modified Postgate B and the bacteria were harvested in the late exponential phase corresponding to a decrease in sulphate concentration from about 2000 mg/L to 400 mg/L, taking into account that a sulphate concentration below 400 mg/L revealed to be limiting for bacterial growth.

Since in some experiments, FLX was expected to be the only carbon source it was necessary to perform previously a washing step to eliminate the carbon source (lactate). For this purpose, the

enriched inoculum was centrifuged at 2500 ×g for 10 min at room temperature, the supernatant was discarded, and the pellet re-suspended using the Postgate B without lactate or MSM. This procedure was repeated twice.

2.3. Biodegradation of FLX

Anaerobic cultures were prepared to evaluate the performance of the obtained bacterial community in terms of resistance and degradation of FLX.

The cultures were performed using 10% (v/v) of the previously enriched inoculum on three different media spiked with FLX:

(i) MSM, a mineral salt medium without carbon compounds which was composed by 0.5 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.01 mg/L NaCl, 0.2 g/L $MgCl_2.6H_2O$, 0.02 g/L $CaCl_2$, 0.0004 mg/L $ZnSO_4.7H_2O$, 0.0004 g/L $CoCl_2.6H_2O$, 0.0003 mg/L $MnSO_4.H_2O$, 0.01 mg/L EDTA and 0.0003 mg/L $(NH_4)_6Mo_7O_{24}.4H_2O$) spiked with 50 mg/L FLX;

(ii) a modified Postgate B medium used to growth SRB, composed by 0.5 g/L KH_2PO_4, 1 g/L NH_4Cl, 1 g/L Na_2SO_4, 1 g/L yeast extract, 0.1 g/L ascorbic acid, 0.1 g/L thioglycolic acid, 2 g/L $MgSO_4.7H_2O$ and 7.75 g/L of the carbon source $C_3H_5O_3Na$ – sodium lactate and spiked with 20, 50 and 100 mg/L FLX;

(iii) a modified Postgate B medium without sodium lactate as carbon source but spiked with 20 and 50 mg/L FLX as sole carbon source.

All media were autoclaved (120ºC for 45 min) and cooled to room temperature before the addition of FLX and inoculation. A stock solution of 1000 mg/L FLX was added to the media using a PES syringe filter of 0.2 μm pore size from VWR (Leuven, Belgium) to achieve the required drug concentrations. The tested concentrations used were above those found in the environment aiming to study the resistance of bacteria to this drug and its biodegradation, since the few studies within the literature examining the effects of FLX on free-living microorganisms suggested low acute toxicity to bacteria (Oakes *et al*. 2010). Munoz-Bellido *et al*. (2000) reported that a minimum inhibitory FLX concentration of 32 mg/L was required to exert antimicrobial activity against *Corynebacterium*, and bacterial abundance in microcosms did not appear to be adversely impacted by pharmaceutical mixtures that included FLX (Richards *et al*. 2004). Other point is that if bacteria can degrade these higher concentrations, thus more effective may be the removal at much lower concentrations. This can be stated since in a medium rich in carbon sources such as wastewater the bacteria may prefer the carbon source most available and easy to degrade, but according to the BioWin predictive model at these high concentrations it can be inferred that bacteria may be able to use the drug as carbon and energy source even in a complex medium (Çeçen and Gül, 2020).

Negative controls (without sludge inoculum) were performed with all tested media to evaluate the eventual degradation of these compounds by other ways than the biological, or to check eventual interactions of the drug with the medium. Moreover, to evaluate the possible adsorption of FLX on the sludge used as inoculum, independent assays were performed with autoclaved/inactivated (120ºC for 45 min) sludge, in Postgate B spiked with 20 and 50 mg/L FLX and in MSM media with 50 mg/L of this drug.

All the assays were carried out in triplicates, in dark conditions (to avoid photodegradation) at room temperature and at atmospheric pressure. To create anaerobic conditions, a layer of liquid paraffin was added to cultures, acting as a barrier to prevent oxygen exchanges and the batch flasks were sealed with butyl rubber stoppers and aluminium crimp seals to avoid air intake into the flask.

The experiments in modified Postgate B were maintained for 31 days, more than the time to assure high SRB activity and total reduction of the sulphate in the growth media, according to the experience of the research group with these bacteria, namely for acid mine drainage bioremediation purposes (Costa and Duarte, 2005; Da Costa *et al*. 2013; Vitor *et al*. 2015). The assays in MSM, a non-specific medium for SRB growth, were performed and maintained for 28 days, in order to verify which bacteria from the consortium other than the SRB could be able to use FLX as a source of carbon. The formation of metabolic products was also analysed during the degradation process.

The summary of experimental set-ups used to evaluate FLX biodegradation is presented in the **Table 5.1.**

Table 5.1 Summary of the assays used to evaluate FLX biodegradation

	Conditions	**Carbon sources**		**Inoculum source**	
Growth media	**For Respiration**	**Lactate (mg/L)**	**FLX (mg/L)**	**Autoclaved inoculum**	**Anaerobic sludge (Faro's East WWTP)**
Modified Postgate B	Anaerobic	7750	-	-	✓
Modified Postgate B	Anaerobic	7750	20	✓	✓
			50	✓	✓
			100	✓	✓
Modified Postgate B	Anaerobic	NT	20	✓	✓
			50	✓	✓
MSM	Anaerobic	NT	50	✓	✓

✓ = tested; - = not added

Aiming to evaluate the SRB activity, the cultures in Postgate B were monitored for pH and redox potential (Eh) using a GLP 21 pH meter, Crison (Barcelona, Spain) and for the sulphate concentration through molecular UV/Visible spectroscopy at 450 nm using a Hach-Lange™ DR

2800 spectrophotometer (Sköndal, Sweden) and the sulfaVer4 method from Hach-Lange (Düsseldorf, Germany). The optical density (OD) measurements of microbial growth were performed at 600 nm in a Hach-Lange™ DR 2800 spectrophotometer (Sköndal, Sweden).

One-Way ANOVA (Single Factor) tests using Excel Data Analysis Tools were performed to evaluate the differences between the cultures with inoculum in the absence or presence of the drug and the correspondent negative control (without or with inactivated inoculum), with a significance level (α) of 0.05.

2.4. Quantification of FLX by High Performance Liquid Chromatography (HPLC) analysis

Immediately after collecting the samples, they were filtered with 0.2 µm polypropylene (PP) syringe filters from VWR (Leuven, Belgium) and then stored overnight at 4 ºC before chromatographic analysis.

Fluoxetine was analysed using a modular Advanced Scientific Instrument KNAUER HPLC system with a Smartline UV detector 2600 Smartline Manager 5000 (Berlin, Germany). The output signal was monitored and integrated using ClarityChrom® software. The compounds were separated using a reversed phase XbridgeC18 column (4.6 × 250 mm, 5 µm particle size) - Hybrid technology connected to a Guard Column Xbridge-C18 column (4.6 × 20 mm, 5 µm particle size), both purchased from Waters Corporation (Milford, MA, USA).

To analyse the biodegradation of FLX an HPLC analysis was performed using an isocratic method with a mobile phase consisted of ACN:water (75:25, v/v) adjusted to pH 2.87 with orthophosphoric acid (85%) using a flow rate of 1.0 mL/min and a total run time of 7 minutes with the column maintained at room temperature. The injection volume was 20 µL and a wavelength of 204 nm was used for detection.

Specific standard calibration curves were constructed for each experiment to determine the concentrations of FLX in the corresponding samples. The concentration ranges of FLX standards prepared with the respective medium were from 0.1 to 100 mg/L of the drug.

The limits of detection (LOD) were determined based on the series of measurement results for the standard solutions with the three lowest concentrations. A LOD of 0.26 mg/L FLX was estimated. Based on the relationship LOQ (limit of quantification) = 3×LOD, the obtained LOQ was 0.78 mg/L FLX. The obtained working range was of 0.78÷100 mg/L.

2.5. Identification of FLX metabolic products by Gas Chromatography–Mass Spectrometry (GC-MS)

The identification of NFLX and other potential metabolic products that could possibly be generated from the biodegradation of FLX was performed by GC-MS.

Anaerobic cultures in Postgate B medium spiked with 20 and 50 mg/L FLX, as unique carbon source and the negative control of 50 mg/L FLX (the highest concentration used), after 28 days of assay, were pooled, respectively and sample fractions of 5 mL were collected by elution in 100% ACN at pH 5 using a VARIAN 380-LC Evaporative Light Scattering Detector (ELSD) coupled with HPLC. Samples were dried at 50 ºC with nitrogen during 60 min, and dissolved in 100 µL methanol to inject in the GC-MS.

GC-MS detection of FLX and NFLX: The column was a Restek-Rxi® - 5 Sil MS (Crossbond®, similar to 5% diphenyl/95% dimethylpolysiloxane) 30-meter, 0.25 mm ID, 0.25 µm df). Instrument parameters were as follows: Injection volume 1 µL, injector temperature, 250 ºC in mode splitless, oven temperature programming was initial temperature 160 ºC, hold for 2 min, 9 ºC/min to 260 ºC, hold for 5 min and 260 ºC to 300 ºC at 20 ºC/min, hold for 5 min at final temperature, flowrate 1 mL/min, transfer line 250ºC, source 220ºC, carrier gas helium.

The metabolites were analysed by mass spectral Library using the search program NIST 2014, 10th edition MS search version 2.0.

2.6. Molecular characterization of FLX degrading bacterial communities

The microbial dynamics were studied through a massive sequencing of 16S rRNA genes on anaerobic cultures, in order to identify taxa putatively involved in the degradation of FLX and/or its metabolites. Microbial communities were studied along the anaerobic experiments, in cultures of Postgate B medium with 20 and 50 mg/L FLX and MSM of 50 mg/L of FLX in which the drug was used as sole carbon source. The three replicates correspondent to each experiment, condition and date of culture were pooled together. Thus, to identify microorganisms putatively capable of degrading FLX, DNA was extracted from culture samples with the PowerSoil® DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), which uses bead beating and silica spin filter technology for extraction. The concentration and quality of eluted DNA was determined using a NanoDrop spectrophotometer Thermo Scientific 3300 and DNA samples were sent to the company DNASense ApS (Aalborg, Denmark) for large-scale biodiversity analysis through massive parallel sequencing of 16S rRNA genes according to the following procedures already described in (Palma *et al*. 2018).

2.6.1. 16S rRNA amplicon library preparation

The procedure for bacterial 16S ribosomal ribonucleic acid (rRNA) amplicon sequencing targeting the V1-3 variable regions is based on Caporaso *et al*. (2012) using primers adapted from the Human Gut Consortium (Ward *et al*. 2012). Ten nanograms of extracted DNA was used as template and the polymerase chain reaction (PCR) (25 µL) contained deoxynucleotide (dNTPs) (400 nM of each), $MgSO_4$ (1.5 mM), Platinum® Taq DNA polymerase HF (2 mU), 1X Platinum® High Fidelity buffer (Thermo Fisher Scientific, USA), and barcoded library adaptors (400 nM) containing V1-3 specific primers: 27F AGAGTTTGATCCTGGCTCAG and 534R ATTACCGCGGCTGCTGG. PCR settings: Initial denaturation at 95 °C for 2 min, 30 cycles of 95 °C for 20 s, 56 °C for 30 s, 72 °C for 60 s and final elongation at 72 °C for 5 min.

All PCR reactions were run in duplicate and pooled. The amplicon libraries were purified using the Agencourt® AMpure XP bead protocol (Beckmann Coulter, USA) with the following exceptions: the sample/bead solution ratio was 5/4, and the purified DNA was eluted in 33 µL nuclease-free water. Library concentration was measured with Quant-iT™ HS DNA Assay (Thermo Fisher Scientific, USA) and quality validated with a Tapestation 2200, using D1K ScreenTapes (Agilent, USA). Based on library concentrations and calculated amplicon sizes, the samples were pooled in equimolar concentrations and diluted to 4 nM.

2.6.2. DNA sequencing

The purified sequencing libraries were pooled in equimolar concentrations and diluted to 4 nM. The samples were paired end sequenced (2 × 301bp) on a MiSeq (Illumina) using a MiSeq Reagent kit v3, 600 cycles (Illumina) following the standard guidelines for preparing and loading samples on the MiSeq. 10% Phix control library was spiked in to overcome low complexity issue often observed with amplicon samples.

2.6.3. 16S rRNA amplicon bioinformatic processing (bacteria V1-3)

Forward and reverse reads were trimmed for quality using Trimmomatic v. 0.32 (Bolger *et al*. 2014) with the settings SLIDINGWINDOW:5:3 and MINLEN:275. The trimmed forward and reverse reads were merged using FLASH v. 1.2.7 (Magoc and Salzberg, 2011) with the settings -m 25 -M 200. The merged reads were dereplicated and formatted for use in the UPARSE workflow (Edgar, 2013), The dereplicated reads were clustered, using the usearch v. 7.0.1090 -cluster_otus command with default settings. Operational taxonomic unit (OTU) abundances were estimated using the usearch v. 7.0.1090usearch_global command with -id 0.97. Taxonomy was assigned using the RDP classifier (Wang *et al*. 2007) as implemented in the parallel_assign_taxonomy_rdp.py script in QIIME (Caporaso *et al*. 2010), using the MiDAS database v.1.20 (McIlroy *et al*. 2015). The results

were analyzed in R (R Core Team, 2015) through the Rstudio IDE using the ampvis package v.1.24.0 (Albertsen *et al*. 2015).

The raw massive sequencing data from 16S rRNA gene amplicons generated in this work was archived in the NCBI Sequence Read Archive (SRA) database under SRA study SRP159619 and BioProject PRJNA486835.

3. Results and discussion

3.1. Biodegradation assays of FLX

The anaerobic cultures used in the biodegradation experiments started by an inoculum of a bacterial community from a Faro's East WWTP lagoon system sludge that was previously enriched in modified Postgate B medium under anaerobic conditions for 7 days, in order to favour the growth of SRB. The SRB are a unique physiological group of prokaryotes because they have the ability of using sulphate as the final electron acceptor in respiration (Barton, 1995), that can be found in many reduced environments, such as anaerobic sludge (Kleikemper *et al*. 2002). SRB were found to grow on environmental contaminants such as petroleum hydrocarbon constituents (PHC) (*e.g.*, benzene, toluene, ethylbenzene, xylenes, naphthalene, phenanthrene and alkanes) and halogenated compounds (Ensley and Suflita, 1995; Zhang and Young, 1997; Kleikemper *et al*. 2002; Muyzer, 2008). Thus, in the present work, that particular anaerobic community (SRB) was tested for its FLX biodegradation ability.

The possible occurrence of adsorption was also investigated. All the assays were performed in a batch system.

According to the literature, the biodegradability of FLX is considered very low and the main mechanism described for removal of this drug is adsorption. There are few studies reporting FLX as biodegradable.

3.1.1. Cultures in the presence of sodium lactate and FLX as supplementary carbon source under anaerobic conditions

Initially, FLX removal was studied in anaerobic cultures, where 20, 50 and 100 mg/L of the drug was added simultaneously with 7.75 g/L of sodium lactate (carbon source used in the standard Postgate B medium composition) as described in the **Table 5.1**.

The effect produced by FLX on the growth and activity of SRB consortium was evaluated by measuring parameters such as pH, redox potential, sulphate concentration and optical density (OD) of microbial growth at 600 nm. The results are presented in **Figure 5.2**.

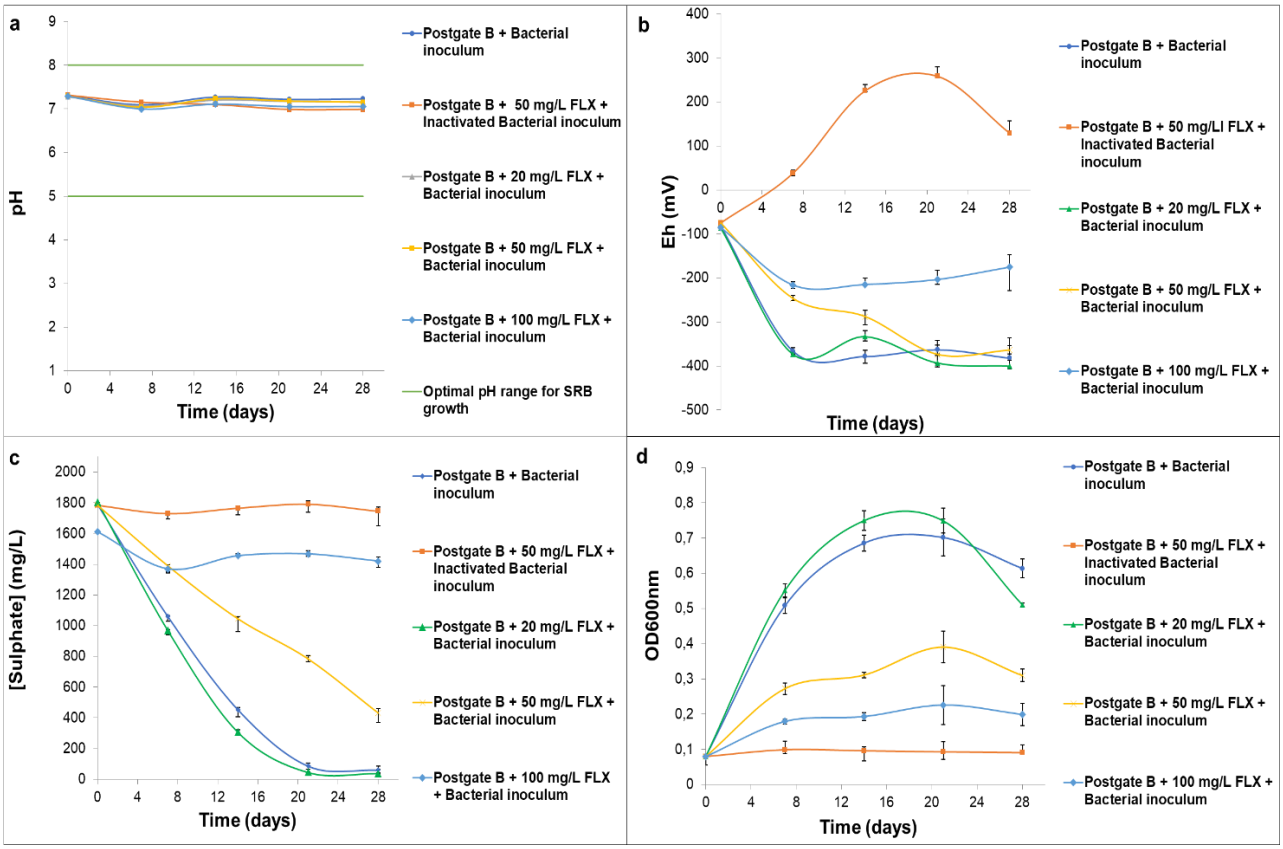

Figure 5.2 Graphical representation of SRB consortium activity parameters: pH (a), redox potential (E_h) (b), sulphate concentration (c), Optical Density at 600 nm (d) in Postgate B medium spiked with 20, 50 and 100 mg/L FLX. Values are expressed as the mean ± standard deviation (n=3)

The pH of SRB cultures media spiked with 20, 50 and 100 mg/L FLX was adjusted to approximately 7. The pH values remained constant throughout the experiment for all the drug concentrations tested (**Figure 5.2a**) and were within the optimum pH range for SRB growth which are between 5 and 8 (Postgate, 1984; Philp *et al.* 1991; Barton *et al*. 1995), as indicated by the horizontal lines of **Figure 5.2a**.

In the cultures of SRB without and in the presence of 20 and 50 mg/L of the FLX, the E_h of the media started around -100 mV and decreased over the course of the days reaching -400 mV, indicating SRB activity and consequent reduction of sulphate into sulphide. In the cultures spiked with 100 mg/L of FLX, the decrease of E_h was up to -200 mV remaining practically constant afterward (**Figure 5.2b**). This is corroborated by a slight decrease in sulphate concentration and is associated to a lower bacterial growth and activity (**Figure 5.2c, d**). In the presence of autoclaved inoculum (negative control) the redox potential increased and remained always above 100 mV, as expected since the bacteria were inactivated.

In SRB cultures spiked with 20 mg/L FLX, the sulphate reduction into sulphide was about 83 ± 1%, therefore significantly faster ($p<0.05$) than in positive cultures without the drug (75 ± 3%), after 14 days of incubation. However, the reduction of sulphate was similar in the 20 mg/L FLX and in the

positive control cultures, with values of about 98.0% ± 0.2% and 97 ± 1%, after 28 days of assay (**Figure 5.2c**). The enhanced sulphate reduction in the presence of 20 mg/L of FLX can be explained by the use of the drug co-metabolically or by the fact that at this concentration the drug can also serve as complementary carbon source for bacterial growth in addition to lactate, as referred by Kleikemper (2002), who described that some SRB were able to use a variety of organic carbon sources, and the enhanced sulphate reduction was concomitant with carbon source degradation (Kleikemper, 2002). Agreeing with the previous results, the highest and fastest bacterial growth occurred at 20 mg/L of FLX, given by an OD_{600nm} of 0.75 ± 0.03, after 14 days, while in the positive control without drug the bacterial growth was of 0.69 ± 0.02, after 14 days (**Figure 5.2d**).

For cultures with 50 mg/L FLX, the sulphate concentration decreased significantly ($p<0.05$) during the assay, with percentages of 41 ± 5%, 52 ± 2% and 76 ± 4%, after 14, 21 and 28 days of experiment, respectively (**Figure 5.2c**). The maximum value of bacterial growth obtained for cultures spiked with 50 mg/L FLX was given by an OD_{600nm} of 0.39 ± 0.05, after 21 days of assay (**Figure 5.2d**).

For cultures with 100 mg/L FLX there was no significant ($p<0.05$) sulphate reduction as indicated by the obtained values: 12 ± 2% after 28 days of incubation, which are in agreement with the OD values: OD_{600m} of 0.23 ± 0.06 after 21 days experiment (**Figure 5.2d**). In the negative control (inactivated inoculum) in which no bacterial activity was expected, there was neither a significant sulphate reduction nor bacterial growth (**Figure 5.2d**). From the results it can be inferred that the SRB bacteria was not resistant to 100 mg/L FLX and that this concentration seems to be toxic to these specific group of bacteria. However, other bacteria resistant to FLX may arise in the community.

Simultaneously with the measurement of the SRB growth and activity parameters, FLX concentrations were analysed during the experiments, in order, to investigate the drug removal by the SRB anaerobic consortium (**Figure 5.3**).

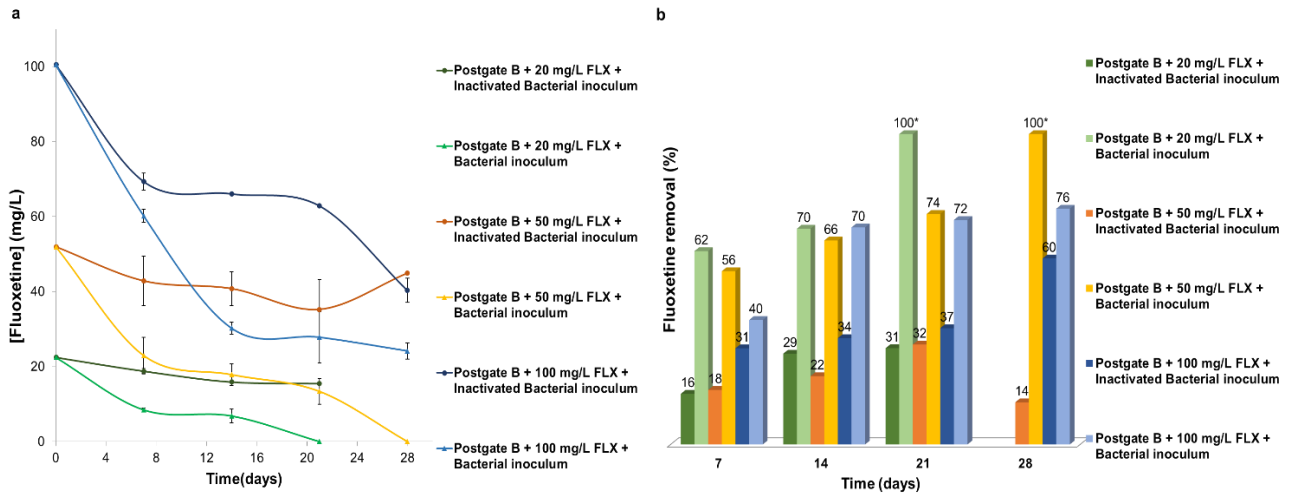

Figure 5.3 Graphical representation of FLX degradation profile by anaerobic bacteria over 28 days of assay (a) and FLX removal in percentage (b). Values are expressed as the mean ± standard deviation (n=3). *100% means that the values of FLX concentration were below the LOD of 0.26 mg/L

For all tested concentrations there was a decrease in the FLX over the course of the experiment, culminating with a removal below the limits of detection for SRB cultures spiked with 20 and 50 mg/L of the drug after 21 and 28 days of experiment, respectively (**Figure 5.3a, b**). The results obtained for cultures spiked with 20 and 50 mg/L FLX showed a significant ($p<0.05$) drug removal when compared with the correspondent negative controls (inactivated inoculum) (**Figure 5.3**). Nevertheless, in the cultures with inactivated inoculum spiked with FLX, appears to have been a contribution of adsorption to the removal of FLX and that adsorption seems to be particularly important in the sample containing 100 mg/L. For example, in the negative control with 50 mg/L FLX the drug removal decreased from 32 ± 4% after 21 days to 14 ± 8% after 28 days of incubation, which may be due to desorption of the drug, or to chemical interactions between FLX and the culture medium compounds (**Figure 5.3b**). Moreira *et al*. (2015) described a system where FLX was removed through adsorption and a mechanism of adsorption and desorption during a reactor operation.

The removal of FLX by the inoculated cultures spiked with 100 mg/L FLX (76 ± 2%) was significantly higher ($p<0.05$) in comparison to the corresponding negative control with inactivated sludge (60 ± 3%), after 28 days of assay. In this case it seems that both, adsorption mechanism and bacterial activity (biodegradation processes) contributed for FLX removal. Although the biodegradation did not appear to have been due to SRB activity, other bacteria belonging to this community may be involved in the removal of FLX, thus, being responsible for the drug's biodegradation.

3.1.2. Cultures in the presence FLX as sole carbon source under anaerobic conditions

Several studies demonstrated that organic compounds can be used either as carbon source and/or as terminal electron acceptors for bacterial growth (*e.g.* Muyzer, 2008; Murphy *et al.* 2008; Seo *et al.* 2009; Kumar and Maitra, 2016; Palma *et al*. 2018). The enriched SRB inoculum was cultured in Postgate B spiked with 20 and 50 mg/L FLX as unique carbon source, in anaerobic conditions, in order to study the ability of this consortium to growth and use FLX. Negative (medium with inactivated sludge) and positive (medium with lactate) controls were also performed (**Table 5.1**). The obtained results are presented in **Figure 5.4 and 5.5**.

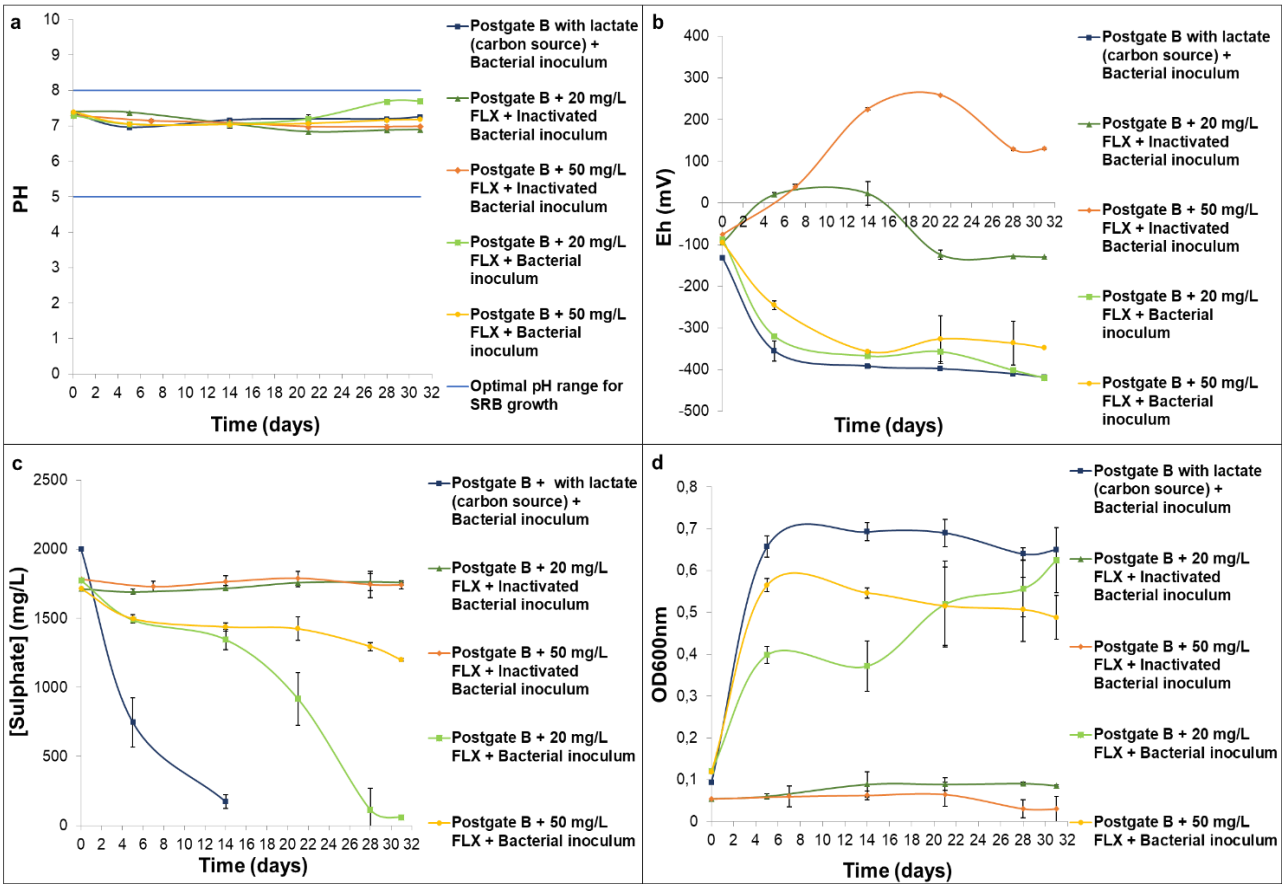

Figure 5.4 Graphical representation of SRB consortium activity parameters: pH (a), redox potential (E_h) (b), sulphate concentration (c), Optical Density at 600 nm (d) in Postgate B medium with 20 and 50 mg/L FLX as unique carbon sources. Values are expressed as the mean ± standard deviation (n=3)

The pH remained neutral (about 7) for all the tested FLX concentrations and positive control (**Figure 5.4a**). The redox potential of the culture media spiked with 20 and 50 mg/L FLX as unique carbon source and of the positive control, evolved from -100 mV to values between -350 and -400 mV, which revealed the SRB activity (**Figure 5.4b**).

For the SRB cultures in the presence 20 mg/L FLX it was verified that the progression of sulphate reduction was slower (completed only after 28 days) than in the positive control, in which the sulphate was completely reduced after 14 days of incubation (**Figure 5.4c**). At this concentration of

FLX the bacterial growth was given by an OD_{600nm} of 0.40 ± 0.02 after 7 days, remaining constant until the 14th day. After that time, there was an increase in the bacterial growth up to OD_{600nm} values of 0.62 ± 0.08 after 31 days of incubation (**Figure 5.4d**). It should be noted that the OD_{600nm} values of the positive control displayed their maximum (0.69 ± 0.02) after 14 days (**Figure 5.4d**). The behaviour showed by the growth curve of the cultures in the presence of 20 mg/L FLX may be due to an adaptation of the SRB consortium to the drug as carbon source.

On the other hand, for cultures spiked with 50 mg/L of FLX the percentage of sulphate reduction was 31 ± 4% after 31 days of experiment (**Figure 5.4c**). Although sulphate reduction was not significant during the experiment, the OD_{600nm} values were of 0.56 ± 0.02 and 0.49 ± 0.05 after 5 and 31 days, respectively, a bacterial growth occurred and remained constant during the assay (**Figure 5.4d**). These results suggest the possible growth of other bacteria than SRB, that belong to the consortium and were able to grow and use that concentration of FLX as carbon source.

In the negative controls the redox potential remained positive or above -100 mV and the OD_{600nm} did not suffer changes remaining very low ($OD_{600nm} \approx 0.1$). Also, the sulphate was not converted along the 31 days of assay (**Figure 5.4b, d**).

As described in the Material and Methods section 2.3., the SRB inoculum was also cultured in MSM, an inorganic medium, spiked with 50 mg/L of FLX used as the only carbon source available (**Table 5.1**). The assay with this non-specific medium was performed to evaluate which bacteria from this community other than SRB could grow in the presence of this drug. In this assay it was possible to observe a bacterial growth of 0.47 ± 0.03 and 0.45 ± 0.07 after 5 and 28 days of incubation. Also, the redox potential decreased from 261 mV to -300 mV revealing that some activity of SRB seems to occur, although the medium was not specific for their growth.

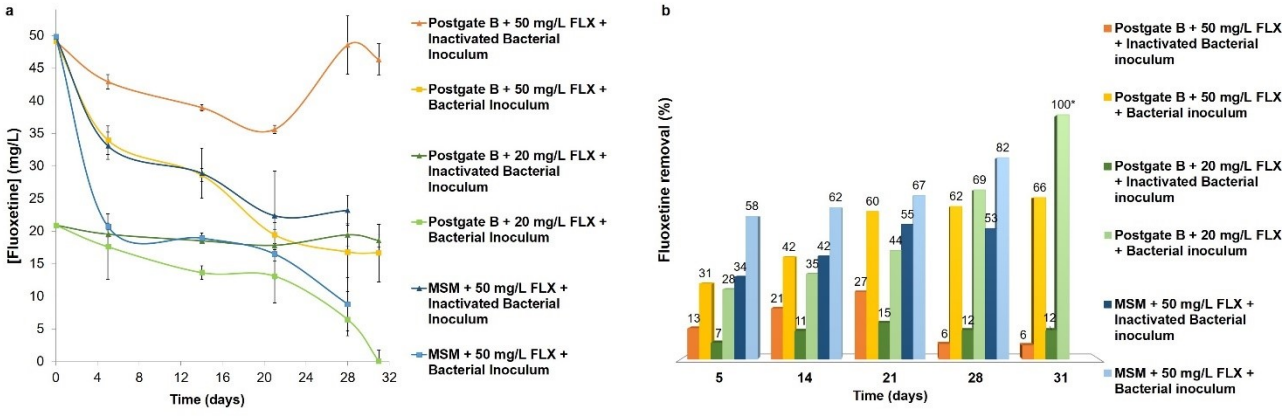

Figure 5.5 Graphical representation of FLX removal by anaerobic bacteria over 31 days of assay. Values are expressed as the mean ± standard deviation (n=3). *100% means that the values of FLX concentration were below the LOD of 0.26 mg/L

In SRB cultures spiked with 20 mg/L FLX the drug removal percentages were of 28 ± 10%, 44 ± 9%, 69 ± 12% and 100 ± 8% (below LOD), while for the inactivated inoculum were of 7 ± 3%, 15

± 2%, 12 ± 4% and 12 ± 5%, after 5, 21, 28 and 31 days of incubation, respectively (**Figure 5.5**). Therefore, for inoculated cultures with 20 mg/L FLX the drug removals increased significantly ($p<0.05$) compared with the negative controls during the course of the experiment, suggesting bacterial degradation of FLX.

Also, for cultures with 50 mg/L FLX the percentages of drug removal obtained for the inoculated tests (31 ± 4%, 60 ± 4%, 62 ± 15% and 66 ± 9%) were significantly higher ($p<0.05$) than in the negative controls (13 ± 2%, 27 ± 1%, 6 ± 4% and 6 ± 5%), after 5, 21, 28 and 31 days of experiment (**Figure 5.5**). These results are in accordance with the ones obtained for bacterial growth and activity, despite the low sulphate reduction at 50 mg/L FLX (**Figure 5.4c, d**).

In the case of the inoculated cultures in MSM spiked with 50 mg/L FLX, the removal of FLX was of 58 ± 1%, thus significantly higher ($p<0.05$) than in the negative control (inactivated inoculum), which was 34 ± 4%, after 5 days of experiment. The removal achieved in the inoculated cultures (67 ± 8%) was not significantly different ($p>0.05$) from the results obtained in the negative control (55 ± 14%), after 21 days of experiment. However, in these cultures the removal was about 82 ± 8%, which is significantly higher ($p<0.05$) compared with the results of the inactivated inoculum (53 ± 5%), after 28 days of assay (**Figure 5.5).** Thus, the results in MSM medium suggest that in addition to biodegradation other mechanisms, such as adsorption to the sludge, or interactions between the drug and medium components, may contribute for FLX removal.

Despite this, it seems that the SRB present in this consortium were not the only bacteria involved in FLX removal, once it was possible to observe bacterial growth and activity in Postgate B cultures even when sulphate was not reduced, as well as in the MSM cultures. As demonstrated by the bacterial identification of 16S rRNA to characterize the populations, other microorganisms than SRB, initially less abundant in the inoculum, may have been directly, or indirectly, responsible for the observed FLX removal.

3.2. Identification of FLX metabolic products by GC-MS analysis

In humans, FLX is metabolized in the liver, being submitted to hepatic metabolism by cytochrome P450 enzymes (CYPs) producing several metabolites by a nonlinear kinetics. FLX is excreted with an unchanged percentage of less than 10%, or as N-desmethylfluoxetine (NFLX), or as glucuronides of both FLX and NFLX. Other produced metabolite is the phenolic compound p-trifluoromethyl phenol (originated via oxidative O-dealkylation by CYPs 2C19 and 3A4) which is inactive. NFLX, generated mainly by CYP2D6 (with contributions from 2C9, 3A4, and other CYPs), is pharmacologically comparable to FLX, yet having a significantly longer half-life ($t_{1/2}$ = 4 to 16 days) (Wenthur *et al*. 2014).

Both FLX and NFLX remain biochemically active in the environment and may present a threat to aquatic species and human health (Fong *et al.* 1998; Brooks *et al*. 2003; Andrés-Costa *et al*. 2017). Considering that FLX, (R,S)-N-methyl-3-phenyl-3-(4-(trifluormethyl)phenoxy)propan-1-amine, is a low molecular weight, fluorinated drug, constituted by two skewed aromatic rings and by methylene units of the methylpropanamine moiety (Wenthur *et al*. 2014) and that the strength of the carbon-fluorine bond conveys stability to fluorinated drugs, they are likely recalcitrant in the environment, or may be partially metabolized to a more toxic metabolite (Murphy, 2016).

Several studies described the conversion and degradation of aromatic and aliphatic organofluorine compounds by bacteria and fungi (Kiel and Engesser, 2015), nevertheless, to occur their mineralization it is necessary to cleave the highly stable C–F bond (dehalogenation reaction). This can occur either directly by the action of a specific dehalogenase enzyme, or indirectly as a result of the formation of an unstable metabolic intermediate (Kiel and Engesser, 2015). Microbial degradation of such kind of compounds, although relatively slow, can be an important contribution to their long-term environmental fate (Parsons *et al*. 2008).

Samples from the experiments with Postgate B spiked with 20 and 50 mg/L FLX and MSM with 50 mg/L FLX, as unique carbon source, using the enriched inoculum from an anaerobic sludge of the WWTP's lagoon system, for which the highest removal of FLX was obtained, were analysed by GC-MS. The metabolic products obtained by the FLX degradation in the presence of this anaerobic consortium are described in the **Table 5.2** and **Figure S4; Annex 3.**

Table 5.2 Metabolic products obtained from the degradation of FLX in Postgate B medium spiked with 20 and 50 mg/L of FLX and MSM with 50 mg/L of the drug inoculated with an enriched inoculum of an anaerobic WWTP's lagoon system sludge, after 28 days of experiment

Negative control (Postgate B + Inactivated sludge + 50 mg/L FLX)		**SRB inoculum + 20 mg/L FLX in Postgate B**		**SRB inoculum + 50 mg/L FLX in Postgate B**		**SRB inoculum + 50 mg/L FLX in MSM**	
Retention time (min)	**Match (1)**	**Retention time (min)**	**Match (1)**	**Retention time (min)**	**Match (1)**	**Retention time (min)**	**Match (1)**
3.162	1-Dodecanol, 3,7,11-trimethyl- ($C_{15}H_{32}O$)	**4.292**	Butylated Hydroxytoluene ($C_{15}H_{24}O$)	**4.294**	7,9-Di-tertbutyl-1-oxaspiro[4,5]de ($C_{17}H_{24}O_3$)	**3.123**	Ethanol, 2-(9,12-octadecadienyloxy ($C_{20}H_{38}O$)
4.297	Butylated Hydroxytoluene ($C_{15}H_{24}O$)	**4.822**	4-Amino-1,5-pentandioic acid ($C_7H_{13}NO_4$)	**4.820**	Phenol, 2,5-bis(1,1-dimethylethyl) ($C_{14}H_{22}O$)	**4.299**	Phenol, 4-(1,1-dimethylethyl)-2-(1 ($C_{11}H_{16}O$)

4.822	2-Fluoro-6-trifluoromethyl benzoic ($FC_6H_3(CF_3)CO_2H$)	**8.310**	Carbonic acid, eicosyl vinyl ester ($C_{23}H_{44}O_3$)	**4.835**	4-Amino-1,5-pentandioic acid ($C_7H_{13}NO_4$)	**4.596**	Phenol, 2,6-bis(1,1-dimethylethyl) ($C_{14}H_{22}O$)
8.231	**Fluoxetine (N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine) ($C_{17}H_{18}F_3NO$)**	**8.651**	2,6,10-Trimethyltetradecane ($C_{17}H_{36}$)	**8.227**	**Fluoxetine**	**4.826**	2,4-Di-tert-butylphenol ($[(CH_3)_3C]_2C_6H_3OH$)
8.339	**Norfluoxetine ($C_{16}H_{16}F_3NO$)**	**8.775**	Hexadecanoic acid, methyl ester ($C_{17}H_{34}O_2$)	**8.281**	**Norfluoxetine**	**11.391**	Hexadecanoic acid, butyl ester ($C_{20}H_{40}O_2$)
8.788	Methyl 8-methyl-nonanoate ($C_{11}H_{22}O_2$)	**9.113**	2,3-dimethyl-5-(trifluoromethyl)benzene-1,4-diol ($C_9H_9F_3O_2$)	**10.813**	Heptadecanoic acid, 16-methyl-, methyl ester (Methyl isostearate) ($C_{19}H_{38}O_2$)	**22.998**	1,2-Propanediol, 3-(octadecyloxy ($C_{21}H_{44}O_3$)
10.820	Methyl stearate ($C_{19}H_{38}O_2$)	**10.813**	Heptadecanoic acid, 16-methyl-, methyl ester (Methyl isostearate) ($C_{19}H_{38}O_2$)	**11.394**	Hexadecanoic acid, 1,1-dimethylether ($C_{16}H_{32}O_2$)	**23.147**	9-Desoxo-9-x-acetoxy-3-desoxy-7.8. ($C_{28}H_{38}O_{10}$)
11.392	Hexadecanoic acid, butyl ester ($C_{20}H_{40}O_2$) – (palmitic acid, butyl ester)	**11.394**	Hexadecanoic acid, butyl ester ($C_{20}H_{40}O_2$)			**23.373**	(2E)-2-Methyl-2-butenoic acid - 1,1-hexanediol ($C_{11}H_{22}O$)
13.283	Hexadecanoic acid, 1,1-dimethyl ether ($C_{17}H_{34}O_2$)	**12.470**	Pentadecane, 2,6,10,14-tetramethyl ($C_{19}H_{40}$)			**23.544**	5-(3,4-Bis[(trimethylsilyl)oxy]-1,5-cyclohexadien-1-yl)-3-methyl-5-phenyl-1-(trimethylsilyl)-2,4-imidazolidinedione ($C_{25}H_{40}N_2O_4Si_3$)

There are few studies about FLX biodegradation, thus the drug degradation pathway can be inferred by analysing other works where bacteria presented the ability to degrade similar fluorinated compounds. Thus, it can be assumed that the bacteria herein identified has the same biodegrading capacity.

Peaks associated to compounds which were assumed to be contaminants were removed, such as for example bis(2-ethylhexyl) phthalate from the plastic material, which appeared at 14.831 min.

Peaks corresponding to FLX and NFLX appeared in negative controls (with inactivated inoculum) in the presence of 50 mg/L after 28 days of assay, suggesting that the medium components can react chemically with FLX in addition to adsorption (6 ± 4%) (**Figure 5.5**) to the inactivated inoculum **(Table 5.2**).

In the SRB cultures spiked with 50 mg/L FLX, besides the presence of FLX which did not suffered complete degradation (38%), its metabolic product NFLX occurred and other peaks corresponding to products such as phenol, 2,5-bis(1,1-dimethylethyl) ($C_{14}H_{22}O$); 4-amino-1,5-pentandioic acid ($C_7H_{13}NO_4$) also appeared (**Table 5.2**). In this case the probable pathway is *via* phenol derivative para-trifluoromethylphenol ($C_7H_5F_3O$) formation, as described by Andrés-Costa *et al*. (2017).

For the cultures in MSM spiked with 50 mg/L it is not possible to infer about the FLX pathway, since neither NFLX nor a fluorinated intermediate nor even another metabolic product already described were detected (**Table 5.2**).

In the cultures inoculated with 20 mg/L FLX although peaks corresponding to FLX and NFLX did not appear, an intermediate compound the 2,3-dimethyl-5-(trifluoromethyl)benzene-1,4-diol ($C_9H_9F_3O_2$) arised with other compounds such as butylated hydroxytoluene ($C_{15}H_{24}O$); a carbonic acid ($C_{23}H_{44}O_3$) and 4-amino-1,5-pentandioic acid ($C_7H_{13}NO_4$).

The product 2,3-dimethyl-5-(trifluoromethyl)benzene-1,4-diol was maybe detected at the same retention time than FLX, in the HPLC analysis of samples at 20 mg/L FLX, since its degradation was 69 ± 12% at 28 days of assay, whereas in GC-MS analysis neither FLX nor NFLX were detected.

In that particular case 2,3-dimethyl-5-(trifluoromethyl)benzene-1,4-diol seems to be a dead-end fluorometabolite for FLX degradation under the tested conditions. That intermediate suggests that the bacteria predominantly use the nonfluorinated ring of the drug as carbon source, as for example, *Rhodococcus sp*. 065240 strain, which can degrade benzotrifluoride yielding trifluoroacetic acid (Murphy, 2016).

Other possibility is that at day 28 the bacterial population with ability to perform the dehalogenation of the C-F bond was still arising and evolving, leading to the later transformation of the intermediate metabolites and subsequent mineralization.

Parsons *et al*. (2008) found that under anaerobic conditions occur the reductive reactions that leads to dehalogenation. Under reduced conditions, reductive dehalogenation is a thermodynamically favourable process, yielding more energy than by reduction of most of other electron acceptors (Parsons *et al*. 2008). Nevertheless, scarce information is available on the dehalogenation of fluorinated compounds. Despite this, it is expected from the organisms that can perform anaerobic degradation of perfluorinated compounds that they would be capable of a hydrogenolytic defluorination rather than dihaloelimination, since hydrogenolysis of, for example, fluorinated

ethanes, yields more energy than difluoroelimination (Parsons *et al*. 2008). Häggblom and Bossert (2003) reported that perhalogenated compounds are usually just degraded under anaerobic conditions by reductive defluorination rather than by aerobic degradation.

However, only few bacteria own fluoroacetate dehalogenase enzyme which belongs to the exclusive class of enzymes known to specifically hydrolyse C–F, yielding fluoride ion and glycolate (Goldman, 1965; Goldman *et al.* 1967; Kurihara *et al*. 2003; Donnelly and Murphy, 2009; Murphy, 2016). Thus, some microorganisms have evolved to use fluoroacetate compounds as a carbon and energy source (Camboim *et al*. 2012) and to bio-transform aromatic compounds such as fluorophenols, fluorobenzene, and fluorobenzoic acids through catabolic pathways (Murphy, 2016). Some authors referred that depending on the position of the fluorine, therefore, the formation of the oxidized intermediates could result in the spontaneous elimination of fluoride ion or in the production of a dead-end fluorometabolite (Harper and Blakley, 1971; Clarke *et al*. 1975; Schreiber *et al*. 1980; Engesser *et al*. 1990; Murphy, 2016).

The pathways of both 20 and 50 mg/L FLX concentrations appeared to be different, suggesting that the microorganisms involved in the biodegradation could be also different.

Considering that the existing studies in the literature were performed under aerobic conditions and these herein presented were carried out with anaerobic cultures and conditions, it is possible that different transformation products originated by other pathways and/or enzymes could be generated. However, Fetzner (1998) identified in several cases that anaerobic catabolic reactions were similar to those occurring aerobically.

The present work has been accomplished with an anaerobic consortium and conditions, and despite the fact that the pathways for fluorinated compounds described in literature consisted mainly in aerobic processes, it could be assumed that the degradation of FLX underwent a similar pathway since several compounds produced during the process were also similar.

Thus, under aerobic conditions, the fragmentation pathway of FLX, a compound with an aromatic-aliphatic ether fragment, and of its metabolic products included the following transformation reactions; an ether cleavage (N-dealkylation) corresponding to a loss of the isopropyl group, oxidation to benzaldehyde, dehydrogenation and production of a phenol derivative and an aldehyde (Tadkaew *et al*. 2011; Velázquez and Nacheva, 2017). As also other reactions that do not involve the amine functional groups may occur, such as O-demethylation, hydroxylation at groups other than the amine-N or the α-C, desaturation, oxidation of a hydroxylated product to a carbonyl or carboxylic acid product, dehydration of an aliphatic alcohol to an alkene, and combinations of several reactions including the decarboxylation involving the amine functional group (Gulde *et al*. 2016).

For example, Engesser *et al*. (1988) studied the co-metabolism of 3- and 4-trifluoromethyl (TFM) benzoates by *Pseudomonas putida* strains, identifying three products, 4-TFM-2,3-dihydro-2,3-dihydroxybenzoate, 4-TFM-2,3-dihydroxy-benzoate and 2-hydroxy-6-oxo-7,7,7-trifluorohepta-2,4-dienoate (7-TFHOD). They observed that biodegradation was poor with an accumulation of 2-hydroxy-6-oxo-7,7,7-trifluorohepta-2,4-dienoate hydrolase (7- TFHOD) by enzymes of p-cymene pathway. In other study with a marine bacterium, Taylor *et al*. (1993) demonstrated mineralization of metatrifluoromethylbenzoates by combining biodegradation and photochemical processes (Taylor *et al*. 1993; Murphy, 2016). The thermophilic bacterium *Bacillus thermolevorans* was able to transform 2- trifluoromethylphenol to 7-TFHOD which was subsequently degraded by sunlight. However, no fluoride ion was detected, apparently the photoexcited molecule was a trifluoromethyl radical that took a proton from water yielding a volatile fluoro-form that was quickly lost to the atmosphere (Murphy, 2016).

For example, several habitats (*e.g*. aquifers, aquatic sediments) contaminated with huge quantities of aromatic pollutants are often anoxic. In these environments, are the anaerobic microorganisms which play the main role in the degradation of aromatic compounds, thus being responsible for the recycling of carbon and sustainable development of the ecosystem. Investigation on anaerobic biodegradation of polycyclic aromatic hydrocarbons (PAHs) are relatively new, with a limited number of preliminary studies, in which were demonstrated the biodegradation of naphthalene, anthracene, phenanthrene, fluorene, acenaphthene and fluoranthene. Thus, detailed information on anaerobic degradation of PAHs and other organic compounds under sulphate-reducing and nitrate reducing conditions is scarce and the degradation pathways, catabolic genes/enzymes and/or regulatory mechanisms remain to explore. However, this is an important and emerging field that is under development in several aspects of biotechnological applications (Ghosal *et al*. 2016).

3.3. Molecular characterization of the selected FLX biodegrading communities and investigation of their dynamics by phylogenetic analysis of 16S rRNA gene

Bacterial DNA was extracted from the promising biodegradation assays corresponding to Postgate B spiked with 20 and 50 mg/L FLX as sole carbon source and MSM cultures with 50 mg/L FLX as carbon source.

The identification of the bacterial communities was performed by 16S rRNA gene sequencing for bacterial identification using a 16S rRNA amplicon bioinformatic processing bacteria V1-3.

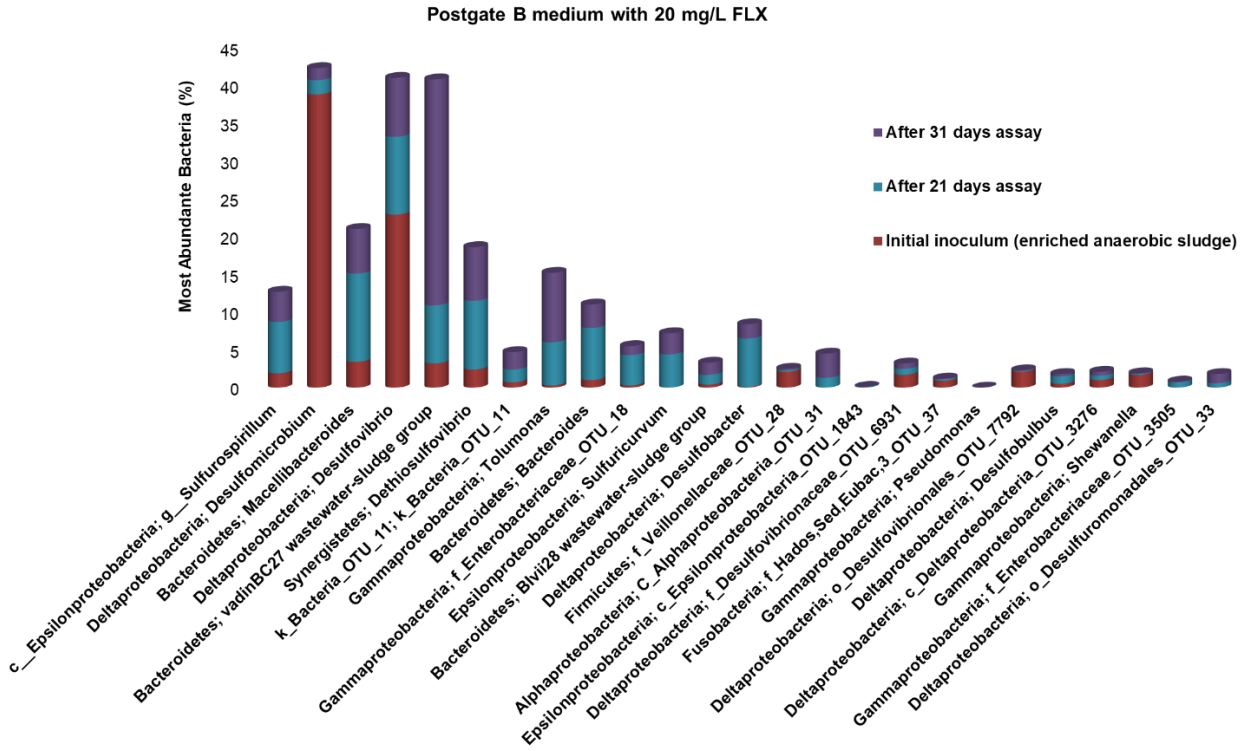

Figure 5.6 The 25 most abundant bacteria (in percent) in samples collected along the experiment with SRB anaerobic cultures using Postgate B spiked with 20 mg/L FLX inoculated with 10% (v/v) of enriched sludge from the WWTP lagoon system. Each bacterium has both a broad group name (phylum) and more specific names (genus and/or family)

The 25 most abundant bacterial groups were given in percentages of 16 S rRNA gene amplicons (**Figure 5.6**). Thus, the initial inoculum from an enriched sulphate-reducing bacteria culture was mainly constituted by two members of the genera *Desulfomicrobium* (38.8%) and *Desulfovibrio* (22.9%) from the class Deltaproteobacteria. During the degradation of 20 mg/L FLX in Postgate B medium, the community suffered a clear shift with a pronounced decrease of the initial population. Thus, after 21 and 31 days of assay the relative abundances of *Desulfomicrobium* were 1.9% and 1.6%, while for *Desulfovibrio* were 10.3% and 7.8%, respectively (**Figure 5.6**). This indicates that *Desulfomicrobium* and *Desulfovibrio* populations were affected by the presence of the FLX as only carbon source.

Over the incubation time of the experiment in the presence of 20 mg/L FLX, a gradual increase in the percentages of 16S rRNA gene amplicons occurred for genera *vadinBC27* wastewater-sludge group, with 3.2% in the initial inoculum to 7.7% and 29.9% after 28 and 31 days of assay, respectively. In addition to *vadinBC27* wastewater-sludge group, also the initial population of *Macellibacteroidetes (3.4%)*, *Dethiosulfovibrio (2.4%)*, *Bacteroides (1%)*, *Desulfobacter (0.1%)*, *Tolumonas (0.3%)*, *Sulfurospirillum (1.9%)*, *Sulfuricurvum (0%)*, f_*Enterobacteriaceae*_OTU_18 (0.3%), *Alphaproteobacteria*_OTU_31 (0%), o__*Desulfuromonadales*_OTU_33 (0%) increased to (11.7%, 9.1%, 6.9%, 6.4%, 4%, 6.8%, 4.4%, 5.7%, 1.3%, 0.6%) and (5.9%, 7.1%, 3.1%, 1.9%,

1.2%, 4%, 2.8%, 9.2%, 3.2%, 1.2%) after 21 and 31 days of experiment, respectively (**Figure 5.6**) suggesting that the representatives of these genera in this population may be involved in FLX and its metabolic products degradation. However, none of these genera is described in the literature as FLX degraders.

In the inoculated cultures spiked with 20 mg/L FLX all the sulphate was reduced. That can be explained by the presence of the resistant and bioactive SRB population mainly composed by *Desulfovibrio, Desulfomicrobium, Desulfobacter* and *Desulfobulbus,* which were initially of 22.9%, 38.8%, 0.1% and 0.5%, whereas after 21 days of experiment their relative abundances were of 19.3%, 1.9%, 6.4% and 1% respectively (**Figure 5.6**). It is important to mention that *Sulfurospirillum* and *Desulfuromonas* are elemental sulphur reducing bacteria*, Dethiosulfovibrio* is a thiosulphate or sulphur reducing bacteria and *Sulfuricurvum,* species can derive energy from the oxidation of elemental sulphur or sulphur reduced compounds (sulphide, sulphite) (Handley *et al*. 2014).

To our knowledge, these bacteria herein identified as the most abundant were not reported in literature as degrading FLX, nevertheless they were described in the literature as having capacity to biodegrade other organic pollutants.

The genus *vadinBC27* wastewater-sludge group was found for the first time in a vinasses anaerobic digester (Godon *et al*. 1997). The genera *vadinBC27* wastewater-sludge group together with *Desulfovibrio*, *Enterobacter* and *Desulfobulbus* could contribute to the decomposition of hardly degradable organic pollutants in landfill leachate (Xie *et al*. 2014), such as nitroaromatic pollutants reducing nitroaromatics to the corresponding aromatic amines (Li *et al*. 2016). However, in addition to this information nothing more is found in the literature about the genus *vadinBC27* wastewater-sludge group, but since its capacity to degrade aromatic compounds was already reported, this genus could be one of the major responsible for FLX decomposition at 20 mg/L FLX.

Zhang *et al*. (2017) hypothesized that *Macellibacteroides* and *Desulfomicrobium* were the key functional genera in anaerobic environments. *Macellibacteroides* and *Desulfomicrobium* were found to work together in WWTPs processes with a high proportion of textile dye wastewater from an industry. Zhang *et al*. (2017) speculated that within sewage treatment plants that treat dye industry wastewater containing aromatic hydrocarbons, the core genera of their anaerobic system would likely consist of aromatic hydrocarbon degrading bacteria, primarily fermentative ones, such as *Macellibacteroides*, and sulphate and sulphur reducing bacteria, such as *Desulfomicrobium*, *Desulfovibrio*, *Desulfobacter, Desulfuromonas, Sulfurospirillum*.

Also*, Tolumonas* which were herein found among the 25 most abundant bacteria, are known for their ability to produce toluene from phenylalanine, phenylpyruvate, phenyl lactate, and phenylacetate, and phenol. Both the presence of a carbon source and the presence of a toluene

precursor were essential for initiation of toluene production, such as butylated hydroxytoluene (Fischer-Romero *et al.* 1996).

Wang *et al*. (2016) found that after the addition of the pharmaceuticals and personal care products (PPCPs) (ibuprofen, naproxen, prednisolone, norfloxacin and sulphamethoxazole), the relative abundance of *Tolumonas* increased from 0.51% to 3.76%. Therefore, they speculated that *Tolumonas* could have a role on biodegradation of antibacterial and anti-inflammatory organic matter (Wang *et al*. 2016).

Giacomucci *et al*. (2012) found that *Desulfovibrio desulfuricans* ATCC 1354, genus herein identified among the most abundant bacteria, was able to degrade nitrocellulose, a binder in paint that is recalcitrant to degradation. Microorganisms can degrade nitrocellulose by two pathways: (i) cleavage of b-1,4-glucoside bonds that produces nitrooligosaccharides of various length, normally carried out by fungi and (ii) nitrocellulose denitration that reduces the degree of nitro substitution, generally performed by bacteria (Giacomucci *et al*. 2012).

There is no description in the literature about degradation of fluorinated compounds by SRB communities, however they revealed to be able to biodegrade other recalcitrant pollutants. Miralles *et al*. (2007) investigated sediments contaminated with a high quantity of oil and found that a wide variety of aliphatic hydrocarbons were significantly biodegraded by bacterial communities after 503 days under environmental conditions at 20 m water depth. They found that shifts in SRB community are a dynamic phenomenon, since bacterial diversity seems to be influenced by the complexity of the mixtures of hydrocarbons. Miralles *et al*. (2007) reported that predominant SRB were affiliated to hydrocarbonoclastic SRB, which can metabolize or transform crude or refined petroleum products, that are undeniably implied in the natural verified cleansing. They also found that *Desulfovibrio-Desulfomicrobium*-like bacteria as several other sulphate-reducing bacteria were capable of oil, alkane or benzene, toluene, ethylbenzene and xylene (BTEX) degradation and to mineralize BTEX and PAHs compounds under anaerobic conditions. However, shorter alkanes (less than C20) were easily degraded than longer alkanes and isoprenoids (Widdel and Rabus, 2001; Miralles *et al*. 2007).

Luijten *et al*. (2003) investigated members of the genus *Sulfurospirillum,* that was herein identified as one of the 25 most abundant genus, and demonstrated that this genus has a broad and diverse substrate range. Ross *et al*. (2016) reported that *Sulfurospirillum spp*. played an important role in sulphur and nitrogen cycling and possesses a metabolic flexibility that enables the reduction of a wide variety of electron acceptors, including thiosulfate, tetrathionate, polysulfide, nitrate, and nitrite. *Sulfurospirillum* are recognized to specifically reduce sulphur and oxidized metals such as arsenate and selenite (Luijten *et al*. 2003). The isolation of strain PCE-M2T, a novel halorespiring (*"*respiring halogenated compounds") species, and the addition of *Dehalospirillum multivorans*

demonstrated the importance of the genus *Sulfurospirillum* in the biotransformation of contaminated soils with chlorinated compounds and metal ions. *Sulfurospirillum halorespirans* can use tetrachloroethylene, selenate, arsenate, nitrate, nitrite, sulphur and fumarate as final electron acceptors (Luijten *et al*. 2003). Thus, anaerobic microorganisms may be able to degrade a wide range of halogenated compounds (Luijten *et al*. 2003).

Starting from the same enriched SRB inoculum used for the experiments in the presence of 20 mg/L FLX, inoculated cultures in Postgate B medium spiked with 50 mg/L FLX as unique carbon source were performed. It was also evidenced that a clear shift occurred from the initial community. In addition, during the experiment these community revealed to be different in terms of genera percentages than the existing during the assays with 20 mg/L FLX (**Figure 5.6 and 5.7**).

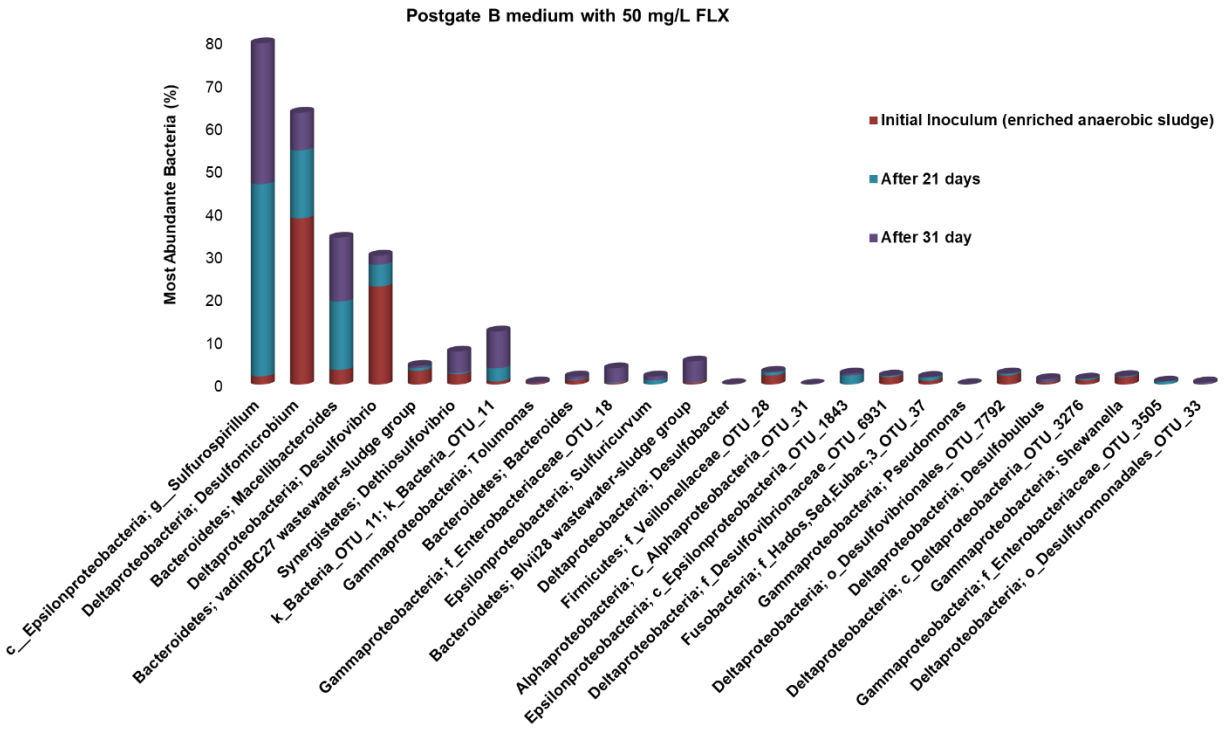

Figure 5.7 The 25 most abundant bacteria (in percent) in samples collected along the experiment with SRB anaerobic cultures using Postgate B spiked with 50 mg/L FLX inoculated with 10% (v/v) of enriched sludge from the WWTP lagoon system. Each bacterium has both a broad group name (phylum) and more specific names (genus and/or family)

Initially, *Desulfomicrobium* (38.8%) was dominant, followed by *Desulfovibrio* (22.9%), whereas the percentages of 16S rRNA gene amplicons for genera *Sulfurospirillum* (1.9%), *Macellibacteroidetes* (3.4%), *Dethiosulfovibrio* (2.4%), *BIvii28* wastewater sludge group an anaerobic fermenter (0.4%), k_Bacteria_OTU_11 (0.7%), f_*Enterobacteriaceae*_OTU_18 (0.3%) became more abundant (*i.e.* 32.9%, 14.9%, 5%, 4.9% 8.6%, 3.3%, respectively) after 31 days of the experiment in the presence of 50 mg/L FLX (**Figure 5.7**). This shift from the initial community

indicates that the SRB community structure was affected by the presence of FLX. Nevertheless, the results suggest that the representatives of these genera in this population may also be involved in FLX and its metabolic products degradation.

Although all the sulphate was converted when 20 mg/L of FLX was used as carbon source, only 24% of sulphate was converted for 50 mg/L of the drug. That can be explained due the existence of a higher percentage of sulphate-reducing bacteria (*e.g. Desulfobacter*) in the community grown in the presence of 20 mg/L FLX than in that grown in the presence of 50 mg/L. In the latter case, a huge reduction of SRB occurred, giving rise to another group of bacteria, the sulphur-reducing bacteria (*e.g. Sulfurospirillum*), which uses elemental sulphur as energetic source. Other explanation may be that the existing SRB bacteria in the presence of such high concentration of FLX became inactive.

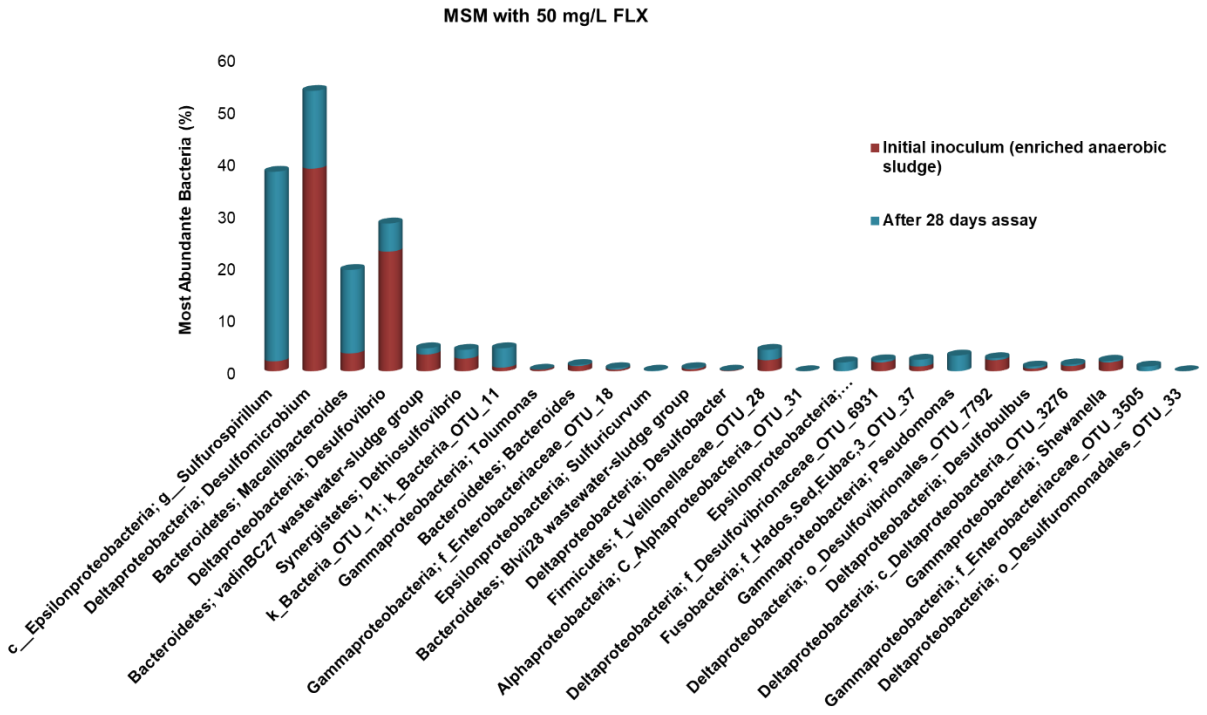

Figure 5.8 The 25 most abundant bacteria (in percent) in samples collected along the experiment with SRB anaerobic cultures using MSM spiked with 50 mg/L FLX inoculated with 10% (v/v) of enriched sludge from the WWTP lagoon system. Each bacterium has both a broad group name (phylum) and more specific names (genus and/or family)

In the assay where MSM medium was inoculated with sulphate-reducing bacteria inoculum in the presence of 50 mg/L FLX, as the only carbon source, once more a clear shift of the initial inoculum occurred (**Figure 5.8**). However, the population presents some similarities to the one inoculated with Postgate B spiked with 50 mg/L FLX. Thus, over the course of the assay there was an increase in the genera *Sulfurospirillum* from 1.9 to 36.3%, *Macellibacteroides* from 3.4 to 16%,

k_Bacteria_OTU_11 from 0.7 to 3.7%, *Pseudomonas* from 0 to 3%, c_Epsilonproteobacteria_OTU_1843 from 0 to 1.7% and finally f_Enterobacteriaceae_OTU_3505 from 0 to 0.9%, after 28 days of incubation (**Figure 5.8**). This also suggests that the representatives of these genera in this population can possibly degrade FLX and its metabolic products.

It was possible to verify that at both 20 mg/L and 50 mg/L FLX a shift occurred in the bacterial community giving rise to identical populations being the most abundant bacteria belonging to the genera *e.g. Sulfurospirillum, Macellibacteroides, Dethiosulfovibrio, k__Bacteria_OTU_11, f__Enterobacteriaceae_OTU_18,* although in varying abundances, which reinforce that these common genera may be responsible for FLX removal (**Figure 5.7 and 5.8**). In the lower concentration of 20 mg/L FLX, *vadinBC27* wastewater−sludge group, *Tolumonas, Bacteroides* and *Desulfobacter* appear to have an important role in FLX removal because they were the most abundant. However, they seem not to adapt well to 50 mg/L FLX, which is suggested by the decrease in their relative abundances (**Figure 5.7 and 5.8**).

To our knowledge, the microorganisms herein identified have never been described in the literature as FLX degrading organisms, although they have been mentioned as having the ability to degrade other recalcitrant organic pollutants.

4. CONCLUSIONS

The results indicate that the studied anaerobic community played a major role in the biodegradation of FLX. The anaerobic SRB consortium cultured in Postgate B with lactate and FLX as an additional carbon source, degraded below LOD, 20 mg/L and 50 mg/L of the drug, after 28 and 31 days, respectively and was able to degrade 76 ± 2% of 100 mg/L FLX in the end of assay. In the experiments where FLX was used as sole carbon source the consortium was able to degrade 20 mg/L FLX below LOD, and removed 66 ± 9% of 50 mg/L of that drug. During the biodegradation process of Postgate B cultures spiked with 20 mg/L FLX as unique carbon source, the metabolite NFLX was not identified but other intermediate metabolite, the 2,3-dimethyl-5-(trifluoromethyl)benzene-1,4-diol (a terpene with antimicrobial activity) was detected, whereas for 50 mg/L FLX, NFLX was identified, after 28 days of assay.

Regarding the evolution of SRB community that displayed the ability to degrade FLX as sole carbon source, the initial population that was mainly constituted by *Desulfomicrobium* and *Desulfovibrio* suffered a shift during the removal of 20 mg/L FLX with an increase of members of the genera *vadinBC27* wastewater-sludge group, *Macellibacteroidetes, Dethiosulfovibrio, Bacteroides, Tolumonas, Sulfuricurvum,* f_*Enterobacteriaceae*_OTU_18 that are assumed for the first time as having a possible role in FLX degradation. It is important to note that these bacteria

were also identified among the most abundant genera together with *Blvii28* wastewater−sludge group and k__*Bacteria*_OTU_11 during the degradation of 50 mg/L FLX as sole carbon source. Although the main mechanism of FLX removal described in the literature is adsorption to sediments or sludge, in the results herein described, biodegradation, under anaerobic conditions, appears to have a major role in the degradation of this drug.

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CHAPTER 6

Aerobic microbial community dynamics and its bacterial isolates in fluoxetine biodegradation experiments

A modified version of this chapter would be published as:

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ABSTRACT

Fluoxetine a widely used antidepressant, considered an emerging pollutant known to be poorly biodegraded. An aerobic community and bacterial isolates were obtained using activated sludge samples from Faro Northwest wastewater treatment plant (WWTP) as inoculum, in order to mimic the real WWTP's biodegradation conditions and to study the use of fluoxetine as unique carbon source, respectively. The results obtained for cultures of raw wastewater inoculated with sludge under highly aerobic conditions showed that about 89% of fluoxetine was removed, while in the negative control no significant decrease in the drug was observed, after 144 h. During the assay the initial population suffered a shift with an increase in strains of the genera *Aeromonadales*_OTU_14, *Acinetobacter*, *Rheinheimera*, *Shewanella*, *Pseudomonas*, f_*Pseudomonadaceae*_OTU_4914; OTU_24; f_JI49D030_OTU_6171, *Methylobacillus* and *Piscinobacter*, which were likely responsible for fluoxetine biodegradation. Based on 16S rRNA sequences analysis, the six isolates with ability to growth using fluoxetine as unique carbon source, were identified as *Pseudomonas putida*, *Enterobacter ludwigii*, *Pseudomonas nitritireducens*, *Alcaligenes faecalis*, *Pseudomonas aeruginosa* and *Pseudomonas nitroreducens*. *Pseudomonas nitroreducens* showed the highest removal of 55 ± 1% and 21 ± 1% at 20 mg/L and 50 mg/L fluoxetine, respectively after 168 h. Although for all the studied isolates the removal percentages were not very high, the degradation of fluoxetine was significantly higher comparing with the negative control. In the present study biodegradation appears to play the main role on fluoxetine removal and to the best of our knowledge these bacteria are reported for the first time as fluoxetine biodegraders.

Keywords: Fluoxetine; Aerobic bacterial community; Isolates; Biodegradation

1. INTRODUCTION

Nowadays with the advances of the pharmaceutical industry and the massive consume of pharmaceutical drugs by population all over the world, it is verified more and more that these drugs are appearing at increasing concentrations in the water sources being that a great concern due to their adverse impacts on aquatic organisms even at a part-per-trillion concentration level (Chen *et al*. 2013; Kristiansson *et al*. 2011; Li and Zhang, 2010; Wang *et al*. 2018).

Selective serotonin reuptake inhibitors (SSRIs), which comprise a common group of antidepressants and their bioactive metabolites, are included in the group of emerging pollutants that have been found in surface waters, ground waters, and marine receiving waters of sewage treatment plants, as a repercussion of the lack of capacity of wastewater treatment plants (WWTPs) to adequately remove these chemicals (Vasskog *et al*. 2008; Ramirez *et al*. 2009; Amador *et al.* 2018).

Fluoxetine or (R,S)-N-methyl-3-phenyl-3-(4-(trifluoromethyl)phenoxy)propan-1-amine (**Figure 6.1**) is the active compound of Prozac and belongs to SSRIs class, being one of the most commonly prescribed antidepressant in the world. Fluoxetine (FLX) hydrochloride is indicated mainly for treatment of depression, panic, anxiety, or obsessive-compulsive symptoms. This pharmaceutical drug is a member of (trifluoromethyl)benzenes, an aromatic ether and a secondary amino compound consisting of 4-trifluoromethylphenol in which the hydrogen of the phenolic hydroxy group is replaced by a 3-(methylamino)-1-phenylpropyl group (ChEBI, 2019).

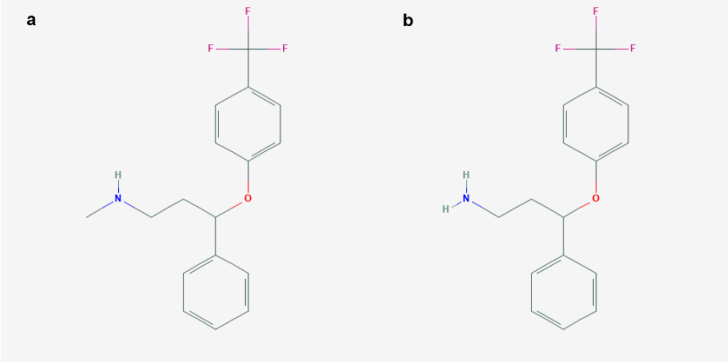


Figure 6.1 Molecular structure of (R,S)-N-methyl-3-phenyl-3-(4-(trifluoromethyl)phenoxy)propan-1-amine commonly known by (a) fluoxetine (ChEBI, 2019) and (b) norfluoxetine (NCBI, PubChem Database, 2019)

Fluoxetine is also a chiral fluorinated pharmaceutical constituted by R- and S- enantiomers, where (R)-FLX was found to be more potent than the S-isomers (Stevens and Wrighton, 1993). Fluoxetine is mainly metabolized by humans to norfluoxetine (NFLX) (**Figure 6.1**) by CYP450N-demethylation and to several other metabolites such as FLX glucuronide, NFLX glucuronide (Benfield *et al.* 1986; Andrés-Costa *et al*. 2017).

The high consumption, long half-life, and the fact that FLX is resistant to biodegradation leads to it being found in aquatic environments such as sewage effluents, surface waters and water resources (Kwon and Armbrust, 2006, Metcalfe *et al*. 2010; Petrie *et al.* 2014). Fluoxetine can be found in high concentrations which can range from 17 ng/L to 3.6 µg/L in polluted waters (Shi *et al*. 2019).

In Portugal, FLX was only detected in the influent wastewaters, with a frequency of 5%, in mean detected levels of 127.97 ng/L (14.6 mg/day/1000 inhab.) (Silva *et al*. 2014).

Both FLX and NFLX have been detected in receiving waters at concentrations ranging from ng/L to µg/L (Andrés-Costa *et al*. 2017). Benotti *et al*. (2009) found that United States tap water was contaminated with 0.64 ng/L FLX and 0.77 ng/L NFLX.

Several studies suggested that waterborne FLX and NFLX remain biochemically active in the environment and can easily accumulate in aquatic organisms through bioaccumulation, which subsequently exerts sublethal physiological effects on these organisms and poses a potential threat to the aquatic ecosystem (Fong *et al.* 1998; Brooks *et al*. 2003; Brooks, 2014; Silva *et al*. 2015; Andrés-Costa *et al*. 2017; Shi *et al*. 2019). For example, FLX and NFLX were found in the tissue of fish collected near municipal wastewater discharges (Andrés-Costa *et al*. 2017). These molecules often act by mimicking the effects of the neurotransmitter serotonin, which regulates a wide range of physiological systems in fish, molluscs, and protozoans, and, even at picomolar levels, have remarkable effects, many of which are unidentified, on these and other aquatic organisms (Fong *et al.* 1998; Silva *et al*. 2012). Changes of the biological activity of aquatic organisms, behavioural problems, reproduction reduction, abnormalities in embryo development, delay in physiological development, anatomical alterations, sexual maturation and immunotoxic impacts were described (Santos *et al*. 2010; Silva *et al*. 2014; Weinberger and Klaper, 2014; Shi *et al*. 2019). Foran *et al*. (2004) reported that FLX was apparently the most toxic pharmaceutical reported, with a No Observed Effect Concentration (NOEC) of 5 µg/L for the fish *Oryzias latipes*. Also, Desbiolles *et al*. (2018) refers that this class of pharmaceuticals appears as the most hazardous, with some ecotoxicity values lower than 1.7 mg/L. FLX can generate potential adverse effects in 50 to 75% of river samples.

The current literature suggests that FLX and NFLX are recalcitrant to hydrolysis, photolysis, and microbial degradation. Both compounds may persist in environmental sediments to where they are rapidly removed from surface waters by adsorption (Cartwright *et al*. 2010; Kinney *et al*. 2006; Kwon and Armbrust, 2006; Moreira *et al*. 2015; Peake *et al*. 2015).

Also, Kinney *et al*. (2006) reported adsorption of FLX to biosolids produced by wastewater treatment plant. Cartwright *et al*. (2010) reported that FLX was efficiently removed (90%) in an activated sludge treatment by adsorption to sludge and not by biodegradation. Moreira *et al*. (2015)

studied FLX adsorption to aerobic microbial granules and proposed the development of adsorption/desorption mechanisms for the removal of this drug from wastewater.

Despite all the studies and harmful effects already known there is still doubts regarding all the environmental risks caused by FLX and its metabolites, being necessary to search for new, effective, economic and ecological ways to degrade or remove them from wastewater treatments.

Microorganisms and particularly bacteria have already demonstrated to play an important role on the biodegradation of organic compounds (Pieper and Reineke, 2000). Thus, the present study focuses on the search for aerobic bacterial communities and isolates, with capacity to biodegrade FLX, since the previous studies mentioned in the literature refer that its removal occurs mainly by adsorption. For that purpose, a bacterial community and isolates were obtained from sludge of an oxidation ditch, an aerobic process, of Faro's Northwest WWTP. The biodegradation of FLX by an aerobic bacterial community was tested using raw municipal wastewater (MWW) under aerobic conditions in order to mimic WWTP's conditions. In the case of bacterial isolates FLX degradation was tested using a mineral salt medium (MSM) where the drug was the only carbon source added.

2. MATERIAL AND METHODS

2.1. Chemicals

The following reagents were purchased from Panreac (Barcelona, Spain): ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$)), calcium chloride ($CaCl_2$), cobalt(II) chloride hexahydrate ($CoCl_2.6H_2O$), manganese(II) sulfate ($MnSO_4$), magnesium chloride hexahydrate ($MgCl_2.6H_2O$), sodium chloride (NaCl), zinc sulfate ($ZnSO_4$). Ethylenediamine tetraacetic acid (EDTA), potassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) both AnalaR NORMAPUR® were obtained from VWR Prolabo Chemicals (Leuven, Belgium). Bacteriological agar was acquired from Panreac (Barcelona, Spain). Ringers solution and peptone were obtained from VWR Prolabo Chemicals (Leuven, Belgium). Glucose, Nutrient Broth for general cultivation, Plate Count Agar (PCA) for standard method Agar were purchased from Himedia Laboratories Pvt. Ltd. (India). Glycerol, BioReagent ≤ 99% purchased from Sigma-Aldrich. The solutions of fluoxetine hydrochloride (99% purity), norfluoxetine hydrochloride (99% purity) were purchased from Sigma-Aldrich (Deisenhofer, Germany). Acetonitrile (ACN) and methanol, both HPLC grade, and phosphoric acid (85% purity) were supplied by VWR Prolabo Chemicals (Fontenay-sous-Bois, France).

2.2. Inocula source and preparation

An activated sludge collected from Faro Northwest WWTP's oxidation ditch (aerobic sludge) was used as inoculum in order to test the biodegradation of FLX mimicking real WWTP's conditions, under aerobic conditions.

For bacterial isolates experiments, 1 g of sludge was rinsed with a Ringer's solution to eliminate all dissolved carbon compounds to ensure that FLX was the only available carbon source for bacterial growth. For this purpose, the inoculum from the aerobic sludge was centrifuged at 2500x *g* for 10 minutes at room temperature, then the supernatant was discarded, and the pellet was subsequently used in successive dilutions. The washing procedure was repeated twice.

2.3. Biodegradation of fluoxetine

2.3.1. Bacterial community experiments

Aerobic cultures (highly aerated) were prepared to evaluate the performance of the different consortium in terms of resistance and degradation of FLX, whilst mimetizing the conditions in the WWTP's oxidation ditch. The raw MWW was collected at the influent inlet of the Faro Northwest WWTP.

The cultures were performed using 10% (v/v) of activated sludge inoculum that was directly inoculated in the raw MWW medium spiked with FLX. A stock solution of 1000 mg/L FLX was added to the media using a PES syringe filter of 0.2 μm pore size from VWR (Leuven, Belgium) to achieve 20 mg/L in the medium. The concentrations used to study the resistance of bacteria to that drug and its biodegradation are above those found in the environment, thus if bacteria can resist and degrade higher concentrations it can be inferred that better will be the removal of residual concentrations (from ng/L to pg/L) of the drug, according to the predictive BioWin model. Munoz-Bellido *et al*. (2000) reported that a minimum inhibitory FLX concentration of 32 mg/L was required to exert antimicrobial activity against *Corynebacterium*, and bacterial abundance in microcosms did not appear to be adversely impacted by pharmaceutical mixtures that included FLX (Richards *et al*. 2004).

Negative controls were carried out to evaluate the possible adsorption of FLX on the sludge used as inoculum and to evaluate the eventual degradation of the drug by other ways than the biological, or to check potential interactions of the FLX with the medium. These assays were performed with autoclaved (120ºC for 45 min) sludge and raw MWW spiked with 20 mg/L of this drug.

All the assays were carried out in batch triplicates, in dark conditions (to avoid photodegradation) at room temperature and at atmospheric pressure.

To achieve aerobic conditions with high oxygenation levels (from 96 to 98% of dissolved oxygen), cultures were carried out in open batches with aeration (110 mL/min airflow). In the experiments

under aerobic conditions the percent saturation of dissolved oxygen (DO) was measured by a portable CD650 meter (Eutech Instruments).

The experiments in MWW were incubated during 144 h.

One-Way ANOVA (Single Factor) tests using Excel Data Analysis Tools were performed to evaluate the differences between the cultures with inoculum in the absence or presence of the drug and the correspondent negative control (with inactivated inoculum), with a significance level (α) of 0.05.

2.3.2. Bacterial isolates experiments

The pellet resulting from the sludge previously washed was incubated in 9 mL of 0.1% peptone water at 28 °C for 2 hours, and a successive dilutions protocol was performed with 9 mL of broth in each tube. Subsequently, 100 µL of each dilution was spread on solid plate cultures with mineral salt medium (MSM) a medium without carbon compounds which was composed by 0.5 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.01 mg/L NaCl, 0.2 g/L $MgCl_2.6H_2O$, 0.02 g/L $CaCl_2$, 0.0004 mg/L $ZnSO_4.7H_2O$, 0.0004 g/L $CoCl_2.6H_2O$, 0.0003 mg/L $MnSO_4$, 0.01 mg/L of EDTA and 0.0003 mg/L $(NH_4)_6Mo_7O_{24}.4H_2O$), with a nitrogen source of 1 g/L $(NH_4)_2HPO_4$ and agar (15 g/L).

All media were autoclaved (120 ºC for 45 min) and cooled to room temperature before the addition of FLX and inoculation.

The experiments were carried out by adding to the solid MSM:

i. 10 g/L glucose as positive control, to monitor bacterial growth, given that this compound is the preferred carbon source for most of the common heterotrophic bacteria (Moat *et al.* 2002);
ii. 50 and 100 mg/L of FLX as unique carbon source.

A screening of bacterial isolates was performed in solid medium in order to select and isolate bacteria resistant to FLX. The selection was done using colony morphotype procedure, which is defined as a group of bacteria exhibiting typical colonial patterns (pigmentation, shape, size, elevation, margin) (Lebaron *et al*. 1998; Fleury *et al.* 2001). Bacteria belong to the same morphotype, if under similar growth conditions, develop colonies of the same morphology for certain growth conditions. Colonial growth patterns of a morphotype are inherited and can be generated following inoculation with a single cell (Fleury *et al.* 2001). The selected colonies with ability to grow in the presence of FLX were picked for further identification and to perform FLX biodegradation experiments. The cultures were incubated in the dark at 28 ºC for 24, 48 and 144 h.

The obtained isolates were stored in 30% (v/v) of glycerol at -20 ºC.

Biochemical tests were performed, catalase activity was determined by adding 3% (v/v) of hydrogen peroxide solution, a positive reaction is indicated by the production of bubbles and

oxidase activity was evaluated with Microbact oxidase strips (OXOID). The gram staining protocol was accomplished as described by Gram (1884) and Cowan and Steel (1965) and observed on a light microscope (Leica Microsystems).

The bacteria which grew in the presence of FLX and subsequently isolated were grown in PCA medium for further use. Liquid cultures were carried out to study the biodegradation of FLX with a dilution of 1:300 (v/v) of each bacterial isolate that displayed the ability to grow in the presence of FLX. After bacterial isolates identification, the further experiments were carried out in liquid medium using the MSM performed as described above supplemented with a nitrogen source of 1 g/L of $(NH_4)_2HPO_4$ and a carbon source: 10 g/L glucose (positive control) and spiked with 20 and 50 mg/L of FLX. Negative controls only with the drug and in the absence of the bacterial isolates were performed. The bacterial isolate cultures were incubated at 28 °C, 150 rpm in the dark. The sampling was after 24, 48 and 168 h, depending on the bacterial grow. The assays were performed in triplicate. The bacterial growth was measured by optical density (OD) at 600 nm in a Hach-Lange™ DR 2800 spectrophotometer (Sköndal, Sweden).

One-Way ANOVA (Single Factor) tests using Excel Data Analysis Tools were performed to evaluate the differences between the cultures with inoculum in the absence or presence of the drug and the correspondent negative control (without inoculum), with a significance level (α) of 0.05.

2.4. Effect of fluoxetine on chemical oxygen demand (COD) degradation for biodegradation experiments using a bacterial community

COD (mg O_2/L) is a measure of water and wastewater quality. The COD test is often used to monitor WWTP efficiency, quantifying the amount of oxidizable pollutants found in its influents, effluents and in the receiving waters.

The COD was analysed in assays with MWW medium in the presence and absence of 20 mg/L FLX to evaluate its contribution for COD and the influence of this drug on the degradation of the organic matter present in wastewater. COD was also determined in the MWW medium assays with 20 mg/L FLX without inoculum, to evaluate the variation of COD caused by adding this drug and by its removal. For that purpose, samples were collected immediately after the preparation of these experiments and after 144 h incubation.

COD measurements were carried out using cuvette tests for the dichromate method with LCK 514 kit, purchased from Hach-Lange (Düsseldorf, Germany), following the procedure described in Palma *et al*. (2018).

2.5. High Performance Liquid Chromatography (HPLC) analysis

Immediately after collecting the samples, they were filtered with 0.2 µm polypropylene (PP) syringe filters from VWR (Leuven, Belgium) and then stored overnight at 4 ºC before chromatographic analysis.

FLX and NFLX was analysed using a modular Advanced Scientific Instrument KNAUER HPLC system with a Smartline UV detector 2600 Smartline Manager 5000 (Berlin, Germany). The output signal was monitored and integrated using ClarityChrom® software.

The compounds resulting from experiments with:

- bacterial communities were separated using a reversed phase XbridgeC18 column (4.6 × 250 mm, 5 µm particle size) - Hybrid technology;
- bacterial isolates were analysed by reversed phase C18 Spherisorb ODS2 Column (80Å, 5 µm, 4.6 mm × 250 mm) purchased from Waters Corporation (Milford, MA, USA).

The columns were connected to a Guard Column Xbridge-C18 column (4.6 × 200 mm, 5 µm particle size), all of them were purchased from Waters Corporation (Milford, MA, USA).

2.5.1. Quantification of fluoxetine

To analyse the biodegradation of FLX an HPLC analysis was performed using an isocratic method:

i. For bacterial community the mobile phase consisted of ACN:water (75:25, v/v) adjusted to pH 2.87 with orthophosphoric acid (85%) using a flow rate of 1.0 mL/min and a total run time of 7 minutes with the column maintained at room temperature.

ii. For bacterial isolates the mobile phase was 10 mM of triethylammonium phosphate:ACN (40:60, v/v) adjusted to pH 3.5 with orthophosphoric acid (85%) using a flow rate of 1.0 mL/min and a total run time of 10 minutes with the column maintained at room temperature. The injection volume was 20 µL and a wavelength of 204 nm and 226 nm was used for detection.

Standard calibration curves were constructed for each experiment to determine the concentrations of FLX in the corresponding samples. The concentration ranges of FLX standards prepared with the respective medium were from 0.1 to 100 mg/L of the drug.

The limits of detection (LOD) were determined based on the series of measurement results for the standard solutions with the three lowest concentrations. A LOD of 1.06 FLX was estimated for MWW. Based on the relationship LOQ (limit of quantification) = 3×LOD, the obtained LOQ was 3.19 FLX for MWW. The obtained working range was of 3.19÷100 mg/L.

2.6. Characterization of fluoxetine degrading bacterial communities

The microbial dynamics was studied through a massive sequencing of 16S rRNA genes on aerobic cultures, in order to identify taxa putatively involved in the degradation of FLX and/or its metabolites. Microbial communities were studied along the aerobic experiments, in cultures where the close to real conditions of WWTPs were mimetized. The three replicates correspondent to each experiment, condition and date of culture were pooled together. Thus, to identify microorganisms supposedly able of degrading fluoxetine, DNA was extracted from culture samples with the PowerSoil® DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's protocol. The concentration and quality of eluted DNA was determined using a NanoDrop spectrophotometer Thermo Scientific 3300 and DNA samples were sent to the company DNASense ApS (Aalborg, Denmark) for large-scale biodiversity analysis through massive parallel sequencing of 16S rRNA genes. The procedures for 16S rRNA amplicon library preparation, DNA sequencing and 16S rRNA amplicon bioinformatic processing (bacteria V1-3) are described in Palma *et al*. (2018).

The raw massive sequencing data from 16S rRNA gene amplicons generated in this work was archived in the NCBI Sequence Read Archive (SRA) database under SRA study SRP159619 and BioProject PRJNA486835.

2.6.1. Identification of fluoxetine degrading bacterial isolates

DNA of each isolate was extracted from cultures incubated at 28 ºC in the dark after 48 h using the E.N.Z.A.® Soil DNA kit, following the protocol provided by manufacturer.

PCR amplification of the bacterial 16S rRNA gene conserved region the following universal primers were used the 8F (*forward*) 5'-AGAGTTTGATCCTGGCTCAG-3'/ 1492R (*reverse*) 5'-TACCTTGTTACGACTT-3'. The reaction mixture consisted in 2 µL of DNA sample, 1 µL of primer 8F, 1 µL of primer 1492R, 2.5 µL of Dream Taq Buffer, 0.1 µL of Dream Taq DNA polymerase and 0.5 µL dNTPs mixture (NZytech), with a final volume of 25 µL of reaction. Amplifications were performed on the thermal cycler 2720 (Applied Biosystems) using the following conditions: initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 1 minute (denaturation), 57 °C for 30 seconds (primer annealing) and 72 °C for 2 minutes (elongation), and final elongation at 72 °C for 5 minutes. The gel electrophoresis was carried out using 5 µL of amplified PCR product to which it was added 2 µL of loading dye buffer x6 (NZYtech) containing blue and orange bromophenol dyes G on an agarose gel (1% agarose, Tris-1% Acetate-EDTA (TAE)). 3 µL of DNA safe SYBR (NZYtech) was added to the agarose gel. As molecular marker, 5 µL of 1 kb NZYDNA Ladder VII (NZYtech) was used, in order to estimate, by comparison, the size of the analyzed DNA fragments. The migration was conducted at 100 V for 1

hour. The gel was examined in a UV transilluminator (Biorad Universal Hood II Gel Doc System, UK) for visualization of the DNA fragment bands.

2.6.2. DNA sequencing

The amplification of 16S rRNA gene generated a PCR product of 1484 bp. The sequencing reaction by the Sanger method was carried out using the purified PCR product (template amount of about 40 ng of DNA) with *primer*s 8F/1492R and the *BigDye Terminator Cycle sequencing* v*3*.1. kit (Applied Biosystems, USA) (with a labeling dye for each of the nucleotides). The POP-7 polymer (Applied Biosystems, USA) was used as separation matrix in order to perform DNA sequencing and fragment analysis in a Genetic Analysis 3130xl sequencer (Applied Biosystems, USA). The results were obtained by Sequencing Genetic Analysis Software, provided by CCMAR's Molecular Biology platform.

The sequence reads analysis were treated using the BIOedit Sequence Alignment Editor and contig-0 sequences were generated as listed in **Table S5** (available in the **Annex 4**). Taxonomic identity was established through Blast search of Gene Bank database of NCBI and to RDP Classifier by RDP Naive Bayesian rRNA Classifier Version 2.11 (Wang *et al*. 2007).

3. RESULTS AND DISCUSSION

3.1. Biodegradation assays of fluoxetine by an aerobic bacterial community

In order to mimic the aerobic FLX biodegradation process that can occur in the WWTP, a sludge collected from the Faro's Northwest WWTP oxidation ditch was used directly as inoculum source. At the same time the possibility of adsorption was considered, thus a negative control using raw wastewater and inactivated inoculum (autoclaved medium and sludge sample) with 20 mg/L of FLX, and with aeration (approximately 96 ± 1% of dissolved oxygen) was performed (**Figure 6.2**).

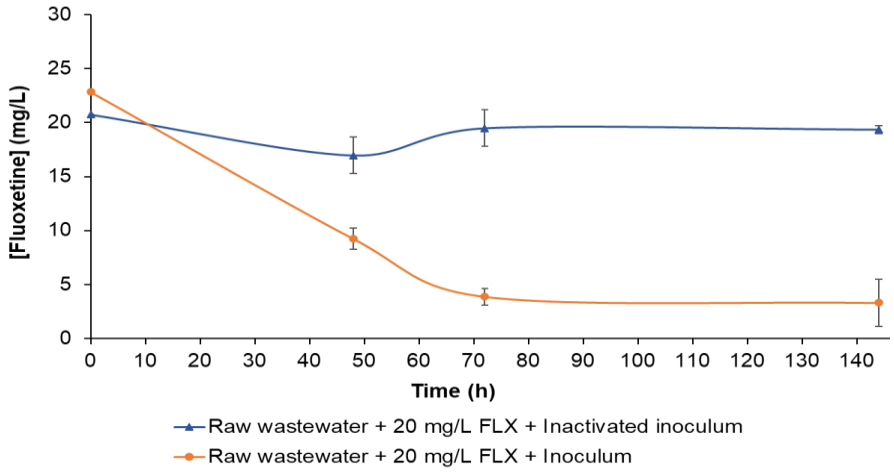


Figure 6.2 Graphical representation of 20 mg/L FLX degradation profile by aerobic bacteria from WWTP oxidation ditch over 144 h of assay. Values are expressed as the mean ± standard deviation (n=3)

FLX and NFLX were detected in almost overlapping peaks varying between 3.7 and 3.86 min, that may occur due to its similar chemical structure. Thus, in the present work we are assuming that the removal of FLX corresponds also to the removal of NFLX. This assumption can be valid since bacteria can degrade directly FLX into other compounds than NFLX and in the case of FLX metabolization into NFLX this metabolite can be subsequently degraded by bacteria.

In the presence of the bacterial community, it was possible to see a decrease in drug concentration throughout the experiment from 23 ± 1 to 3 ± 2 mg/L of FLX, whereas in the case of the negative control the concentration values remained relatively constant after 144 h of assay (**Figure 6.2**). The drug was gradually removed from the medium with removal percentages of 60 ± 4% and 85 ± 3%, after 48 h and 72 h, respectively, values that were significantly higher ($p<0.05$) than those obtained for the negative control, where the removal of FLX was of 18 ± 8% and 6 ± 8% during this period of experiment. The decrease from 18 ± 8% to 6 ± 8% could be due to desadsorption of the drug from the sludge. In the presence of the drug and inoculum the degradation of the FLX was approximately 89 ± 11% after 144 h of assay, whereas in the negative control, where adsorption to the sediments could occur, there was no significant decrease (7 ± 2%) in the drug concentration after the same time (**Figure 6.2**).

The results herein obtained are in accordance with the few descriptions in the literature concerning FLX removal by bacteria, for example Suarez *et al*. (2010) reported transformations of 92% under aerobic and 85% under anoxic conditions of FLX fed at 20 μg/L (0.6 μM) in reactors inoculated with biomass collected from a conventional activated sludge pilot plant (Suarez *et al*. 2010; Moreira *et al.* 2015). Fernandez-Fontaina *et al*. (2012) reported a reduction between 75 and 90% of FLX on a reactor inoculated with nitrifying bacteria. Posteriorly, the authors suggested biodegradation as the main removal process (Fernandez-Fontaina *et al*. 2012; Moreira *et al.* 2015).

However, Moreira *et al*. (2015) studied the degradation of FLX in an aerobic granular sludge-sequencing batch reactor (AGS-SBR) and described that the drug was removed through a mechanism of adsorption and desorption during the reactor operation. Moreira *et al*. (2015) considered that adsorption to aerobic granular sludge can be advantageous for treatment of wastewaters, since the initial biosorption onto the biomass reduces shock loadings. Amorim *et al*. (2016) reported the highest removal efficiency for NFLX, in an AGS-SBR system, where the presence of non-racemic NFLX in the effluent indicated that biologically mediated processes, including biotransformation and/or biodegradation, could be involved in its removal.

3.2. Effect of fluoxetine on chemical oxygen demand (COD)

COD is an important water quality parameter providing an index to determine the effect an effluent will have on the receiving water body. It is therefore a major reference in the control of wastewater discharges. For example, in Portugal, the general COD emission limit value for wastewater discharges is 150 mg O_2/L (Decree-Law n°236/98 of 1 August) and the limit for discharges from urban WWTP is 125 mg O_2/L (Decree-Law n° 152/97 of 19 June) (Palma *et al*. 2018).

The effect of fluoxetine on the removal of COD was also studied. COD was measured in the biodegradation experiments with raw MWW using the sludge from the WWTP oxidation ditch as inoculum in the beginning of the incubation and after 144 h under aeration.

In cultures inoculated in municipal wastewater without drug the initial value of COD was of 358 ± 29 mg O_2/L and after 144 h was 134 ± 1.8 mg O_2/L, whereas in experiments with inactivated sludge in raw municipal wastewater spiked with 20 mg/L FLX (negative control) COD values were of 556 ± 6 and 323 ± 4 mg O_2/L, initially and after 144 h respectively. In the experiment using the sludge as inoculum cultured in wastewater spiked with 20 mg/L FLX the COD was initially of 709 ± 16 mg O_2/L and decreased to approximately 196 ± 8 mg O_2/L after 144 h, suggesting that the degradation of organic matter by the aerobic sludge was not affected by the presence of FLX. Therefore, although COD values decreased in all experiments, COD remained above the regulatory limit at the end of the experiment. The incomplete removal of COD seems not to be only related with the presence of the pharmaceutical, since that fact also occurred also in the positive control (134 mg/L O_2), where FLX was absent.

The incomplete removal of COD can be explained by the presence of organic compounds in the MWW which are slowly degradable and thus, that remained in solution after 144 h incubation. In the case where drug was added these hardly removed compounds could be the intermediate metabolites produced from FLX degradation.

3.3. Molecular characterization of fluoxetine biodegrading communities

In order to explain that obtained results, the bacterial communities were identified by 16S rRNA gene sequencing and the most abundant bacterial groups were given in percentages of 16 S rRNA gene amplicons (**Figure 6.3**).

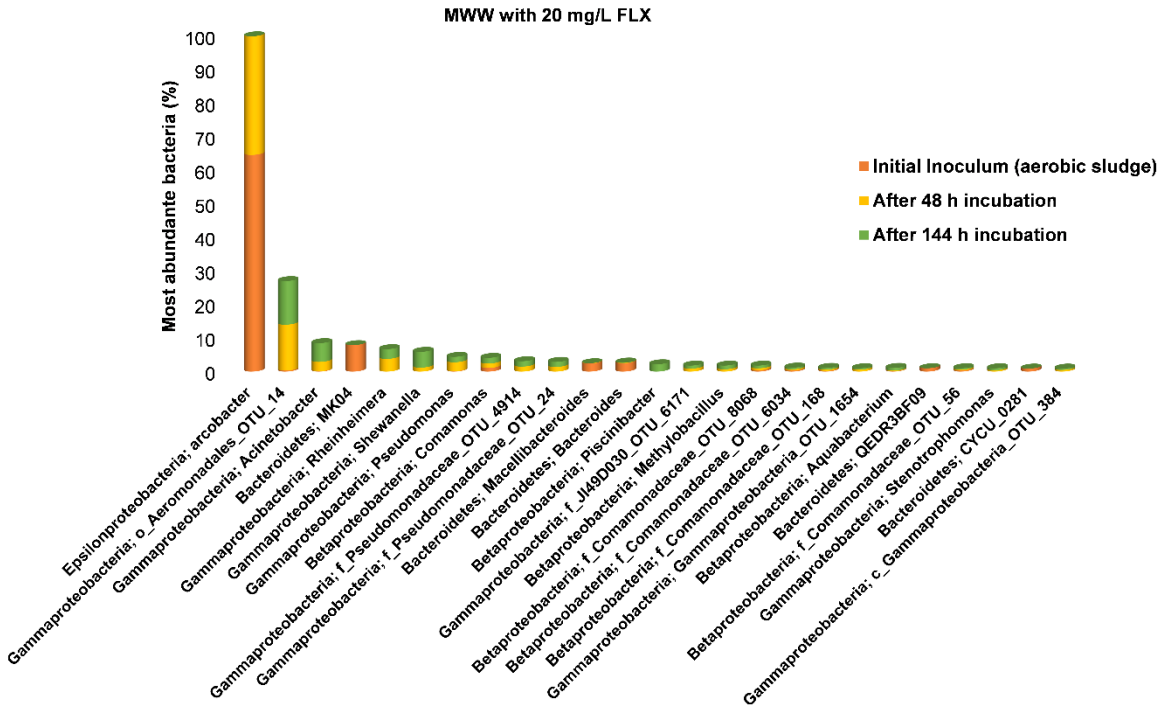


Figure 6.3 The 25 most abundant bacteria (in percent) in samples collected along the experiment with aerated cultures using MWW spiked with 20 mg/L FLX inoculated with 10% (v/v) sludge from the WWTP oxidation ditch. Each bacterium has both a broad group name (phylum) and more specific names (genus and/or family)

The initial inoculum from the municipal wastewater sludge under aerobic conditions was mainly constituted by the genera *Arcobacter* (64.6%), *Bacteroidetes*; MK04 (7.9%), *Bacteroides* (2.5%), QEDR3BF09 (1%), CYCU_028 (0.9%) and *Macellibacteroides* (2.4%). Over the incubation time of the experiment using 20 mg/L of FLX as carbon source and of the subsequent FLX degradation, a clear shift in the bacterial community was recorded. Thus, after 48 h incubation it was possible to verify a decrease in all the genera from the initial inoculum (*e.g. Arcobacter*, MK04, *Bacteroides*, *Macellibacteroides*) and an arising in the percentage of o_*Aeromonadales*_OTU_14 (13.7%), *Acinetobacter* (2.8%), *Rheinheimera* (3.8%), *Shewanella* (1.2%), *Pseudomonas* (2.7%), f_*Pseudomonadaceae*_OTU_4914 (1.5%) and OTU_24 (1.4%), f_JI49D030_OTU_6171 (0.9%), *Methylobacillus* (1.1%), Gammaproteobacteria_OTU_1654 (0.6%) and *Stenotrophomonas* (0.4%). After 144 h incubation, when the drug was almost completely degraded, the species from genera *Acinetobacter* (5.6%), *Shewanella* (4.7%), *Piscinibacter* (2.2%), *Aquabacterium* (0.8%) and *Stenotrophomonas* (0.6%) continued to increase (**Figure 6.3**). The results suggest that the representatives of these genera can possibly degrade FLX and its metabolic products.

According to Moreira *et al*. (2015) a shift in the microbial community of aerobic granules occurred probably due to FLX exposure, which induced adaptation/restructuration of the microbial population. This contributed to the robustness of the reactor, which was able to adapt to the FLX load (Moreira *et al*. 2015).

To our knowledge, the bacteria herein identified as the most abundant were not reported in literature as degrading FLX, nevertheless they were described in the literature as having capacity to biodegrade other organic pollutants. Several examples will be herein referred.

Interestingly o_*Aeromonadales*_OTU_14 are facultative anaerobic organisms mostly found in estuarine waters and fresh water. Within that group there is a strain isolated from the estuary of the River Wear, UK, *Oceanomonas doudoroffii,* capable of degrading phenol in the presence of high salinity (Brown, Sutcliffe and Cummings, 2001).

Shewanella is also a facultative anaerobic genus included in the marine bacteria family Shewanellaceae. Chen *et al*. (2008) found that a *Shewanella* strain NTOU1, originally isolated from the cooling system in an oil refinery, could decolorize and detoxify crystal violet (triphenylmethane dyes) under anaerobic conditions (Chen *et al*. 2008). However, *Shewanella* has been of great interest due to the ability of its species to convert heavy metals and toxic substances (*e.g.* iron, sulphur, uranium) into less toxic products by using them as electron acceptors in certain respiratory situations, making them of interest for environmental clean-up (*e.g.* iron, sulphur, uranium) (Dikow, 2011).

Liu *et al*. (2016) isolated the strain *Acinetobacter calcoaceticus* PA in the group of Gammaproteobacteria from the petrochemical wastewater in South China, this bacterial strain was capable of degrading phenol.

Most members of the genus *Rheinheimera* have been isolated from marine environments. Kumar *et al*. (2015) identified a strain designated IITR-13T, which was isolated from a pesticide contaminated sandy soil from an industrial site in Eloor, Cochin region, southern India.

Also, *Methylobacillus* is a group of methylotrophic aerobic bacteria, that can be found in large numbers in marine and freshwater ecosystems. Kumar and Maitra (2016) studied a newly isolated *Methylobacillus* sp. V29b. which grows on methanol, protocatechuate, monobutyl phthalate, dibutyl phthalate, diethyl phthalate, benzyl butyl phthalate, dioctyl phthalate and diisodecyl phthalate. *Methylobacillus* sp. V29b. degraded 70 % of the initial dibutyl phthalate (DBP) in minimal salt medium and 65 % of the initial DBP in contaminated samples. Four metabolic intermediates, dibutyl phthalate (DBP), monobutyl phthalate, phthalic acid and pyrocatechol, were identified (Kumar and Maitra, 2016).

The genus *Pseudomonas* is known by its ability to degrade a wide number of organic pollutants among them are fluorinated compounds. For example, Harper and Blakley (1971) reported that species of *Pseudomonas* were involved in the degradation of *p*-fluorophenylacetic acid and *p*-fluorobenzoic acid. *Pseudomonas pseudoalcaligenes* KF707 a polychlorinated biphenyl-degrading bacterium that can grow in the presence of various toxic metals and metalloids. *Pseudomonas pseudoalcaligenes* KF707 can use 2- and 4-fluorobiphenyl (a fluoroaromatic compound) as sole

carbon and energy sources. The catabolism of fluorobenzoic acids gave rise to a fluorobenzoate intermediate, which was completely mineralized to fluoride ion, this strongly suggests that the bacterium predominantly uses the nonfluorinated ring of fluorobiphenyl as carbon and energy source (Murphy *et al.* 2008). Donnelly and Murphy (2009) isolated from soil the bacteria *Pseudomonas fluorescens* DSM 8341 that readily degrades fluoroacetate.

Pseudomonas genus can also use as their sole carbon source and energy several PHAs such as naphthalene and also fluorene, which has three rings being the major constituent of fossil fuels and coal derivatives (Seo *et al.* 2009). *Pseudomonas aeruginosa* is an efficient pyrene degrader that can utilize pyrene through induction of high cell surface hydrophobicity (Ghosh and Mukherji, 2016). De Gusseme *et al*. (2011) also identified *Pseudomonas aeruginosa* as a paracetamol degrading bacterium.

Although the relative abundance of *Stenotrophomonas* genus is very low (0.6%) it increased during the incubation time. Deng *et al*. (2015) isolated from sludge and identified *Stenotrophomonas* sp. Strain G, which can efficiently degrade eight organophosphorus pesticides (OPs) and is an excellent candidate for applications in OP pollution remediation.

Concerning *Piscinibacter,* an invention provided a bacterial strain capable of degrading methyl tert-butyl ether, namely *Piscinibacter* sp. MQ-18, and an application thereof to microbial decomposition of the methyl tert-butyl ether. The methyl tert-butyl ether degrading bacterial strain provided by the invention can use methyl tert-butyl ether as a sole carbon source to perform energy propagation and completely mineralize the compound into CO_2 and H_2O (Patent nº CN 106244493A).

As occurred in the anaerobic assays also in the aerobic experiments the investigated microorganisms, to our knowledge, are not described in the literature as fluoxetine degrading organisms, although they have the ability to degrade other recalcitrant organic pollutants.

3.4. Biodegradation assays using bacterial isolates

The bacterial isolates with ability to grow in the presence of FLX, thus which were resistant to drug were selected by colony morphotypes (using the following parameters, colony size, form, color, texture and margin).

The bacterial identification and biochemical characterization were focused on the bacterial isolates that displayed resistance to FLX and which could possibly play a role in the drug biodegradation.

The nearly complete 16S rRNA gene sequences of the resistant bacterial isolates were determined (1484 bp). The sequence reads obtained by Sanger sequencing were treated using the BIOedit Sequence Alignment Editor and the generated contig-0 sequences (**Table S5** available in the **Annex 4**) were analyzed through a BLAST search of Gene Bank database of NCBI (**Table 6.1**). RDP database was also used in order to verify the accuracy of the identification.

Isolates were identified based on maximum obtained similarity scores that were mostly above 98%, which indicates good matching and reliability.

From this experiment it was possible to successfully select six bacteria with ability to grow in the presence of FLX as sole carbon source, two isolates that grew in the presence of 50 mg/L of FLX and four in the presence of 100 mg/L FLX (**Table 6.1**).

Table 6.1 Bacterial identification using 16S rRNA gene sequences of the six bacterial isolates from an aerobic sludge, with ability to grow in the presence of FLX as unique carbon source

Isolate	From cultures	Specie (NCBI Blast)	Query cover (%)	Identity (%)	Accession nº (NCBI Blast)
1	50 FLX	*Pseudomonas putida strain NBRC 14164*	98%	99.21%	NR_113651.1
2	50 FLX	*Enterobacter ludwigii strain EN-119*	100%	99.01%	NR_042349.1
3	100 FLX	*Pseudomonas nitritireducens strain WZBFD3-5A2*	99%	99.22%	NR_133020.1
4	100 FLX	*Alcaligenes faecalis strain NBRC 13111*	100%	98.28%	NR_113606.1
5	100 FLX	*Pseudomonas aeruginosa strain DSM 50071*	97%	99.42%	NR_117678.1
7	100 FLX	*Pseudomonas nitroreducens strain NBRC 12694*	98%	98.01%	NR_113601.1

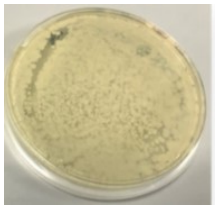
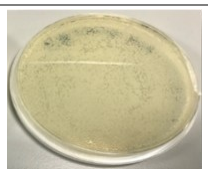
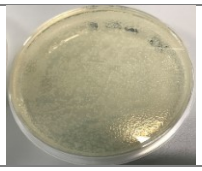
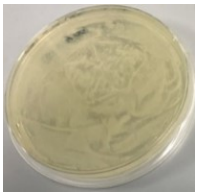
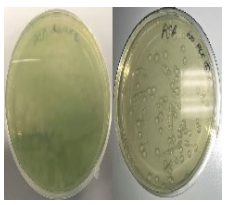
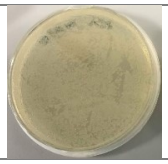
Thus, based on the analysis of 16S rRNA sequences by NCBI BLAST database, the *isolate 1* was identified as *Pseudomonas putida* (type strain NBRC 14169) (GenBank accession NR_113651.1) sharing 99.21% of similarity with this strain; *isolate 2* presented a high similarity of 16S rRNA sequences (99.01%) with a strain identified as *Enterobacter ludwigii* strain EN-119 (GenBank accession NR_042349.1); *isolate 3* share 99.22% of similarity with a strain identified as *Pseudomonas nitritireducens* strain WZBFD3-5A2 (GenBank accession NR_133020.1); *isolate 4* was identified as *Alcaligenes faecalis* with a similarity of 98.28% (GenBank accession NR_113606.1); *isolate 5* identified as *Pseudomonas aeruginosa* strain DSM 50071 (GenBank accession NR_117678.1) with 99.42% of similarity and *isolate 7* sharing 98.01% similarity with *Pseudomonas nitroreducens* strain NBRC 12694 (GenBank accession NR_113601.1) (**Table 6.1**).

According to RDP classifier all the bacterial isolates belong to the phylum "Proteobacteria". The isolates 1, 3, 5 and 7 were classified as Gammaproteobacteria belonging to Order: Pseudomonadales, Family: Pseudomonadaceae and genus *Pseudomonas.* The isolate 2 was classified as Gammaproteobacteria belonging to Order: Enterobacteriales, Family: Enterobacteriaceae and genus *Enterobacter.* The isolate 4 was classified as Betaproteobacteria

belonging to Order: Burkholderiales, Family: Alcaligenaceae and genus *Alcaligenes.* At a genus level all the obtained results were confirmed by RDP database.

The biochemical tests, oxidase and catalase were performed on the *Pseudomonas putida, Enterobacter ludwigii, Pseudomonas nitritireducens, Alcaligenes faecalis, Pseudomonas aeruginosa* and *Pseudomonas nitroreducens.* Several characteristics of the six selected isolates were displayed in the **Table 6.2.**

Table 6.2 Characteristics of the six bacterial isolates which were selected in the presence of 50 and 100 mg/L of FLX

Isolate	Bacteria	Oxidase test	Catalase test	Gram stain	Colony Color	Colony characteristics in PCA	
1	*Pseudomonas putida*	+	+	-	Light yellow	Smooth, shiny, cream, circular and convex. Production of yellowish pigment	
2	*Enterobacter ludwigii*	-	+	-	White	Slightly iridescent or flat with irregular edges	
3	*Pseudomonas nitritireducens*	+	+	-	Pale yellowish	Occurring singly or in pairs	
4	*Alcaligenes faecalis*	+	+	-	Transparent white	Margin usually entire, flat to low convex, spreading irregular edge	
5	*Pseudomonas aeruginosa*	+	+	-	Light green	Produce diffusive greenish fluorescent pigment, large, smooth with flat edges	
7	*Pseudomonas nitroreducens*	+	+	-	Pale yellowish	Smooth, circular, entire, raised	

All the isolates were gram negative, motile, non-spore forming. Comparing the culture, morphological, biochemical characteristics with molecular identification of isolates we can infer that the bacteria belong to presented species of *Pseudomonas, Enterobacter* and *Alcaligenes* genus. For example, cultures inoculated with *Pseudomonas aeruginosa* presented a greenish diffusible colour. *Pseudomonas aeruginosa* is described in literature as producing a soluble bluish-green pigment named pyocyanin/pyoverdine (Kang *et al*. 2018; El-Fouly *et al*. 2015) (**Table 6.2**).

The six bacterial isolates, initially obtained, were further cultured in MSM containing 20 and 50 mg/L of FLX, since concentrations above 50 mg/L FLX revealed to be toxic for bacteria in the liquid cultures.

At the concentration of 20 mg/L FLX, the maximum bacterial growth (OD_{600nm}) observed was very low, up to 0.0270 ± 0.0005, while bacteria which grew in the presence of 50 mg/L of FLX, reached their maximum growth at optical density of 0.0250 ± 0.0006. Despite this very low and slow bacterial growth in the presence of FLX which can be explained as an adaptation of bacteria to the drug as only carbon source, it was possible to detect drug removal during the time of the experiment, indicating that these bacteria may use FLX as a readily degradable carbon source. This may also suggest that FLX degradation does not depend on bacterial concentration but there are some other factors that influence this effect. For example, doubling time of species even within the same genus is not the same, some bacteria grow faster, therefore they deplete carbon sources faster, reach steady state and stop replicating (Allen and Waclaw, 2019).

Moreira *et al*. (2014) described the enantioselective degradation of 2 μM FLX as sole carbon source by *Labrys portucalensis* strain F11 in 30 days. Also, they reported that bacterium growth was about 0.25 after 7 days of experiment, even when FLX was supplemented with acetate (Moreira *et al*. 2014). Other cases of slow bacterial growth were reported in literature as reported by Maksimova *et al*. (2018) where the bacterial growth did not start before complete transformation of acrylamide into acrylic acid.

The results obtained for FLX removal in the biodegradation experiments with cultures of bacterial isolates spiked with 20 mg/L and 50 mg/L FLX are represented in the **Figure 6.4**.

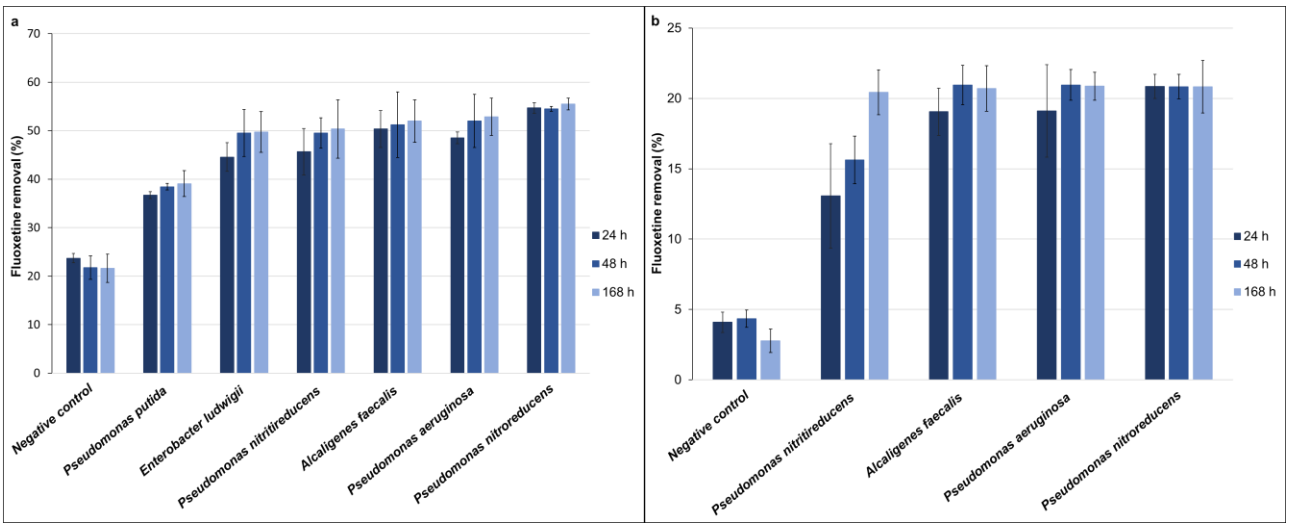


Figure 6.4 Graphical representation of drug removal (%) at 20 mg/L (a) and 50 mg/L (b) of FLX by aerobic bacterial isolates over 168 h of assay. Values are expressed as the mean ± standard deviation (n=3)

All the studied isolates presented similar values of FLX removal at 20 mg/L of the drug after 24 h and 48 h. Thus, *Pseudomonas putida*, *Enterobacter ludwigii*, *Pseudomonas nitritireducens*, *Alcaligenes faecalis*, *Pseudomonas aeruginosa* and *Pseudomonas nitroreducens* degraded 38.4 ± 0.7%, 50 ± 5%, 50 ± 3%, 51± 7%, 52 ± 5% and 54.5 ± 0.5%, respectively of 20 mg/L of FLX values that were significantly higher ($p<0.05$) than the negative control (22 ± 2%) after 48 h (**Figure 6.4a**). Cultures of *Pseudomonas putida*, *Enterobacter ludwigii*, *Pseudomonas nitritireducens*, *Alcaligenes faecalis, Pseudomonas aeruginosa* and *Pseudomonas nitroreducens* in the presence of 20 mg/L FLX showed a removal of the drug of 39 ± 2%, 50 ± 1%, 50 ± 2%, 52 ± 2%, 53 ± 1% and 55 ± 1%, respectively after 168 h. Although for all the studied isolates the removal percentages were not very high, the degradation of FLX was significantly higher ($p<0.05$) comparing with the negative control (**Figure 6.4a**).

Cultures of *Pseudomonas nitritireducens, Alcaligenes faecalis, Pseudomonas aeruginosa* and *Pseudomonas nitroreducens* at 50 mg/L FLX removed 20 ± 2%, 21 ± 1%, 21 ± 1% and 21 ± 1%, respectively after 168 h. The removal percentages were similar throughout the experiment. Although at 50 mg/L of FLX, the maximum of FLX degradation achieved was 21 ± 1% after 168 h, the drug removal was significantly higher in the presence of bacterial isolates (**Figure 6.4b**).

The removal of FLX by bacterial isolates was not complete. Several explanations can be given to this fact:

i) Bacteria work better as a community rather than as isolates, as already described by Mukred *et al*. (2008). The effects of synergistic interactions among members of the community can be the main mechanism behind the advantages of consortia over pure cultures in bioremediation processes (Mukred *et al*. 2008; Alexander, 1999). For

example, if one species has the ability to remove the toxic metabolites, that otherwise may hamper/block the microbial activities of the antecedent species, likely that species can degrade compounds that the first ones are able to degrade only partially (Mukred *et al*. 2008; Alexander, 1999). Mukred *et al*. (2008) referred the theory that each member in a microbial community has an important role and may need/depend on the presence of other species or strains to be able to survive (Mukred *et al*. 2008).

ii) The existence of residual nutrients that can exist in the sludge used as inoculum or/and in the raw wastewater medium, but which were not present in the isolate cultures where FLX was the only carbon source. These carbon sources can help in the growth of bacteria that demonstrated to be able to degrade FLX or favoured the ones that likely stablished synergic effects with FLX degrading bacteria, thus improving the removal of FLX.

iii) An additional contact time of the bacterial isolates with the drug can be needed.

In fact, in terms of further use in real biodegradation processes the choice that makes more sense is the use of bacterial communities. The isolates were used to understand better the bacteria that are part of the degradation process influencing the performance of the consortium.

The identification of bacterial isolates agreed with those obtained for the aerobic bacterial community regarding the removal of FLX, since *Pseudomonas* was within the 25 most abundant bacteria in the community that apparently displayed capacity to biodegrade FLX.

Although all the obtained isolates were not described in literature with the ability to degrade FLX they have been reported as having an important role in the degradation of other organic pollutants. For example:

Pseudomonas was already herein described as having a possible role in FLX degradation. As already described *Pseudomonas* including the species herein identified (*e.g. Pseudomonas putida*, *Pseudomonas aeruginosa)* are regarded as one of the most common species of degrading bacteria which has ability to degrade aromatic compounds due to their ability to use more than 80 different carbon sources (*e. g*. Hinteregger, 1992; Fava *et al.* 1995; Murphy *et al.* 2008; Seo *et al.* 2009; De Gusseme, 2011; Ghosh and Mukherji, 2016).

Enterobacter microorganisms are known to degrade a variety of aliphatic and aromatic amides. An *Enterobacter* with ability to use acrylamide as carbon source was isolated from domestic wastewater (Buranasilp and Jittima, 2011). *Enterobacter ludwigii* was able to biodegrade aniline a harmful substance that pollutes the environment and seriously endangers human health (Kafilzadeh and Khezri, 2016) and use chlorimuron-ethyl an herbicide as carbon source (Pan *et al*. 2018).

Maksimova *et al*. (2018) reported *Alcaligenes faecalis* as acrylamide-utilizing bacteria. *Alcaligenes* can consume acrylic acid as a carbon source and accumulate it. *Alcaligenes faecalis* also have

phenol-degrading potential, some strains secreted and accumulated a vast quantity of phenol hydroxylase in physiological phase, which ensured that the cells could quickly utilize phenol as a sole carbon and energy source (Jiang *et al*. 2007). *Alcaligenes faecalis* was also able to biodegrade phenanthrene a polycyclic aromatic hydrocarbon composed of three fused benzene rings (Kiyohara *et al*. 1982).

A. faecalis could degrade ochratoxin A, which is produced by some filamentous fungi, severely impacts human and animal health by contaminating several types of food and feed, efficiently but could not use it as a sole carbon source (Zhang *et al*. 2017).

This study indicates for the first time, to our knowledge, that the bacterial isolates *Pseudomonas putida*, *Enterobacter ludwigii*, *Pseudomonas nitritireducens*, *Alcaligenes faecalis, Pseudomonas aeruginosa* and *Pseudomonas nitroreducens* may be involved in the biodegradation of FLX.

4. CONCLUSIONS

The results indicate that the studied aerobic communities played a major role in the biodegradation of fluoxetine. The aerobic community was able to degrade 89 ± 11% of 20 mg/L FLX after 144 h. In what concerns COD removal, values decreased from 709 ± 16 mg O_2/L to 196 ± 8 mg O_2/L, remaining, however, above the regulatory limit of 125 mg O_2/L. This may be due to slow degradation compounds such as NFLX and other metabolic intermediates produced during FLX biodegradation. The bacterial community was identified through the analysis of 16S rRNA gene sequences, the initial population was mainly constituted by strains of the genera *Arcobacter*, *Bacteroidetes;* MK04, *Bacteroides*; *QEDR3BF09*, CYCU_028 and *Macellibacteroides,* during the assay an evident shift occurred with increase of strains from genera *Aeromonadales_OTU_14, Acinetobacter, Rheinheimera, Shewanella, Pseudomonas, f_Pseudomonadaceae_OTU_4914; OTU_24; f_JI49D030_OTU_6171, Methylobacillus* and *Piscinobacter*, which are reported for the first time as FLX biodegraders.

In the present work it was possible to obtain bacterial isolates from the sludge of the oxidation ditch of the Faro Northwest WWTP (Portugal) previously used in the community experiments, that were able to utilize FLX as unique carbon source, under aerobic conditions. The selected bacterial isolates that showed resistance and ability to grow in the presence of FLX were sequenced by 16S rRNA gene and identified with similarity scores above 98% as *Pseudomonas putida, Enterobacter ludwigii, Pseudomonas nitritireducens, Alcaligenes faecalis, Pseudomonas aeruginosa, Pseudomonas nitroreducens*. The biodegradation assays showed that *Pseudomonas nitroreducens* displayed the highest removal efficiency of FLX at 20 mg/L, with 55 ± 1% degradation. These results agree with those obtained for the studied bacterial community, since *Pseudomonas* were also within the 25 most abundant bacteria that putatively degraded FLX.

Although the removal of FLX is described in literature to occur mainly by adsorption to sediments/sludge, in the results herein described, biodegradation, under aerobic conditions, appears to have a major role in the degradation of this drug.

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CHAPTER 7

Biodegradation of 17α-ethinylestradiol by *Acinetobacter bouvetii*, *Acinetobacter kookii*, *Pantoea agglomerans*, *Shinella zoogloeoides*

A modified version of this chapter would be published as:

Palma, T. L., Shylova, A., Costa, M. C. (2019). Biodegradation of 17α-Ethinylestradiol by bacteria isolated from an aerobic wastewater treatment process. (*in preparation*)

ABSTRACT

17α-Ethinylestradiol (EE2) is a synthetic estrogen which is classified as group 1 carcinogen by the World Health Organization. Together with other endocrine disruptor compounds, EE2 has been included in the surface water European Watch List since it is found in aquatic environments causing severe adverse effects in the ecosystems. Thus, it becomes of high priority to find or improve processes such as biodegradation of EE2 to completely remove this drug from the wastewater treatment plants (WWTPs).

The present study was carried out by using activated sludge samples from the Faro Northwest WWTP to isolate bacteria with ability to degrade EE2 and study its metabolic pathways. Four isolates with ability to grow in the presence of 50 mg/L EE2 were obtained. The analysis of 16SrRNA gene sequences identified the isolated bacteria as *Acinetobacter bouvetii, Acinetobacter kookii, Pantoea agglomerans, Shinella zoogloeoides*. The results of biodegradation assays showed that *Acinetobacter bouvetii*, *Acinetobacter kookii*, *Pantoea agglomerans* and *Shinella zoogloeoides, Pantoea agglomerans* and *Shinella zoogloeoides* were able to degrade 47 ± 4%, 55 ± 3%, 64 ± 4% and 35 ± 4%, respectively of 13 mg/L EE2 after 168 h at 28 ºC. To the best of our knowledge, these bacterial isolates were identified as EE2 degraders for the first time. The degradation of EE2 by these isolates resulted in several metabolites such as mannonic acid 1,4-lactone, 4-hydroxy-2-quinolinecarboxylic acid, 2,3-dihydroxybenzoic acid, (Z)-aconitic acid, 2-pentenedioic acid and 2-butenedioic acid or fumaric acid that is an intermediate metabolite in the TCA (tricarboxylic or citric acid cycle.

1. Introduction

17α-ethynylestradiol is a synthetic hormone derived of the natural estrogen E2 (17β-estradiol) and an estradiol, which is mainly used as a component of oral contraceptives (Stanczyk *et al.* 2013; Aris *et al.* 2014).

17α-ethynylestradiol is a xenoestrogen where the 3-hydroxy steroid (https://pubchem.ncbi.nlm.nih.gov/compound/estradiol) is substituted by an ethynyl group at position 17, whose molecular formula is given by $C_{20}H_{24}O_2$, M_W=296.403 g/mol, **Figure 7.1** illustrates its chemical structure (NCBI, 2019).

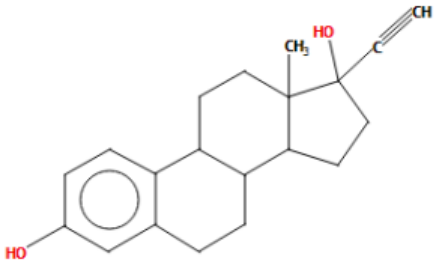

Figure 7.1 Chemical structure of the synthetic estrogen EE2 (NCBI, 2019)

This drug has been shown to exhibit high estrogenic potency on oral administration and can be used in human medicine in estrogen replacement therapy and suspension of breastfeeding (NCBI, 2019; Aris *et al.* 2014).

EE2 belongs to endocrine-disrupting chemicals (EDCs) that constitute a group of organic pollutants with increasing importance due to their impact in the environment and human health (Diamanti-Kandarakis *et al*. 2009). These chemicals may interfere with the function of the endocrine system in wildlife and humans by blocking or mimicking the normal effect of hormones, affecting their synthesis or metabolism, and altering hormone receptor levels (Diamanti-Kandarakis *et al*. 2009).

The human body has three naturally occurring steroid strogens: estrone (E1), 17 β-estradiol (E2), and estriol (E3), with the synthetic estrogen EE2's structure being most similar to that of E2. The potency of these estrogens are typically measured in relation to E2 (having a value of 1) and a re-estimated to have the following relative potencies: EE2: 2; E2: 1; E1: 0.2-0.4; E3: 0.024-0.026. These values have been determined by estrogen receptor binding *in vitro* assays or vitellogenin induction in male juvenile fish (Larsson *et al*. 1999; Thorpe *et al*. 2003; Vanden Belt *et al*. 2004; Williams, 2004; Wise *et al.* 2011).

It is important to note that *in vitro* responses have been known to underestimate *in vivo* responses from more complex wastewater, and potencies can vary somewhat depending on the determination method (Williams, 2004; Wise *et al.* 2011).

17α-Ethinylestradiol is introduced into water by two main routes: excretion and disposal. EE2 is largely excreted by human and animals in urine and faeces as active free forms or inactive glucuronide and sulphate conjugates (Larcher and Yargeau, 2013; Barreiros *et al*. 2016).

The metabolite is subsequently excreted and finds its way to sewage water, where the active EE2 is liberated by hydrolysis. The presence of the ethinyl side chain renders EE2 extremely resistant to natural degradation as well as to sewage water treatment processes (Huang *et al*. 2013; Ternes *et al*. 1999)

Thus, due to the limited ability of the conventional sewage treatment technologies to remove this estrogen, EE2 is steady released via wastewater effluents into surface waters (rivers, estuaries and lakes) (Larcher and Yargeau, 2013; Owen and Jobling, 2012; Cunha *et al*. 2016). For example, Lai *et al*. (2002) reported concentrations up to 62 ng/L of EE2 in WWTP effluent. Zuo *et al.* (2013) detected up to 11.1 ng/L of EE2 in the Lake Quinsigamond, Massachusetts, USA.

EE2 has a high biological activity and was identified as one of the major sources of estrogenic activity in the environment causing negative effects on ecosystems and living beings, even at ng/L levels (Gilbert, 2012; Owen and Jobling, 2012; Larcher and Yargeau, 2013; Cunha *et al*. 2016).

This compound has been associated with the most alarming adverse effects such as fish feminization, the development of female sex characteristics, including female reproductive anatomy with synthesis and secretion of vitellogenin, reproduction and behaviour modifications, fertility reduction (lower the sperm count in males), increase of breast and testicular cancer in humans and promotion of abnormal reproductive processes (*e.g.* Gilbert, 2012; Owen and Jobling, 2012; Avar *et al*. 2016; Bhandari *et al*. 2015; Huang *et al*. 2015; Barreiros *et al*. 2016; Konemann *et al*. 2018).

Moreover, EE2 is the most persistent of the estrogens, with a half-life in water of approximately 17 days, having the potential to bioaccumulate and/or suffer biotransformation and subsequently enter in the food chain (Gomes *et al*. 2004; Atkinson *et al*. 2011; Bhandari *et al*. 2015; Huang *et al*. 2015; Cunha *et al*. 2016). For all these reasons, the European Union has recently added E2 and EE2 to a new "watch

list" of emerging aquatic pollutants included in the Water Framework Directive (Barreiros *et al*. 2016; Konemann *et al*. 2018).

In the case of E2, it is principally a vertebrate hormone, although it was detected in some invertebrates, such as molluscs. Many aquatic species express estrogene receptors. Some species are known to have mechanisms, which allow them to maintain their endogenous hormonal levels in the case of exogenous E2 exposure (Janer *et al*. 2005), but a wide range of species are very sensitive to steroid contaminants (Bhandari *et al*. 2015, Huang *et al*. 2015; Avar *et al*. 2016).

Zuo *et al.* (2013) reported that EE2 was persistent to biodegradation by the lake's microorganisms but that by photodegradation the compound was rapidly removed from the lake surface water under natural sunlight, with a half-life of less than 2 days in summer sunny days. However, the results of lab-scale studies were inconsistent regarding the biological degradation of EE2 by conventional activated sludge samples varying from complete removal to none (Larcher and Yargeau, 2013).

Fewer bacteria were reported has having capacity to biodegrade EE2. For example, Larcher and Yargeau (2013) studied the ability of seven bacterial isolates to degrade EE2 in the presence of ethanol, these were *Pseudomonas putida*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Rhodococcus zopfii*, *Rhodococcus erythropolis*, *Rhodococcus equi* that partially degrade EE2 with efficiencies ranging 21% to 61% and *Rhodococcus rhodochrous* that completely removed the compound after 48 h. Yoshimoto *et al*. (2004) isolated from activated sludge of Japanese wastewater treatment plants, *Rhodococcus zopfii* Y50158 and *Rhodococcus equi* that shown to degrade E1, E2, E3 and EE2. Haiyan *et al*. (2007) reported that *Sphingobacterium* sp. JCR5, a bacterium isolated from an oral contraceptive factory WWTP, was able to grow on EE2 as the sole carbon source. Haiyan *et al*. (2007) proposed an EE2 degradation pathway for *Sphingobacterium* sp. JCR5 in which EE2 is oxidized to E1 followed by cleavage of the B ring and the A ring. Intermediate metabolites were the unsaturated acids 2-hydroxy-2,4-dienevaleric acid and 2-hydroxy-2,4-diene-1,6-dioic acid which were ultimately mineralized to CO_2 and water (Haiyan *et al*. 2007).

Pauwels *et al*. (2008) isolated bacteria from the genera *Phyllobacterium*, *Ralstonia*, *Pseudomonas* and *Acinetobacter* able to co-metabolize EE2 while metabolizing E1 and E2. Shi *et al*. (2002) have isolated from cowshed samples an EE2-degrading microorganism *Fusarium proliferatum* which uses EE2 as the sole source of carbon.

They have reported the existence of EE2-degradative products which may be more polar having a phenolic group, however these substances have yet to be identified.

Some authors found that to a large extent, the natural estrogens were degraded biologically in the denitrifying and aerated nitrifying tanks of the activated sludge system, whereas EE2 was only degraded in the nitrifying tank (Tchobanoglous *et al*. 2000; Vader, 2000; Andersen *et al*. 2003; Dytczak *et al.* 2008).

EE2 was also reported to be degraded completely by nitrifying activated sludge in six days, converting EE2 into hydrophilic compounds. Moreover, studies show that treatment of water with chlorine removes between 80-95% of EE2, and treatment with ozone removes 95-99% of EE2 (Wise *et al.* 2011).

Yet, Owen and Jobling (2012) evoked that the only currently effective option for removing EE2 from wastewater to allow compliance involves adsorption on to granular activated carbon. However, such type of system has unbearable costs, for example for a UK town of around 250,000 people, such a system would cost more than €9 million to install and around €900,000 a year to operate (Owen and Jobling, 2012).

From the need to find new techniques/solutions that are equally effective and at lower costs, comes the bioremediation/biodegradation processes that may represent the most practical and cost-effective mean (Brown *et al*. 2017).

Despite extensive research has already been carried out investigating EE2, there is still indeterminate information regarding its biodegradability, especially the relative change in toxicity and estrogenicity and the identification of by-products (Larcher and Yargeau, 2013; Owen and Jobling, 2012; Brown *et al*. 2017).

Several researchers had work into identification of strains of microorganisms that can break down estrogens with the possible application to wastewater treatment. However, there is little research in the literature on the known degradation pathway or metabolism of estradiol and estriol (Koh *et al*. 2008). Due to the difficulty in biodegrading EE2 and deplete completely the hazardous effect of this compound in aquatic environments it is mandatory to search and identify microorganisms with ability to metabolize EE2 and to clarify metabolic processes that can result in the loss of its estrogenic activity.

Thus, the aim of the present work was to find and investigate bacteria isolated from activated sludge collected from WWTP (Faro, Portugal) that can use EE2 as unique carbon source and the identification of the metabolic products resulting during the drug degradation.

2. MATERIAL AND METHODS

2.1. Inoculum source and preparation

Sludge sample collected from the oxidation ditch (aerobic process) of the Northwest WWTP of Faro (Portugal) was used to obtain bacteria in order to test their resistance and ability to degrade EE2, under aerobic conditions.

After collection, 1 g of sludge was rinsed with a Ringer's solution to eliminate all dissolved carbon compounds to ensure that EE2 was the only available carbon source for bacterial growth. For this purpose, the inoculum from the aerobic sludge was centrifuged at 2500× *g* for 10 minutes at room temperature, then the supernatant was discarded, and the pellet was subsequently used in successive dilutions. The washing procedure was repeated twice.

2.2. Enrichment of bacteria from sludge

Successive dilutions protocol was performed with the washed pellet incubated in 9 mL of 0.1% (w/v) peptone water at 28 °C for 2 hours. Dilution series were performed in 9 mL of fresh nutrient by adding 1 mL of stock solution, and from then on 1 mL of each culture was added to 9 mL of fresh medium successively until making up the dilution of 10^{-7} time.

2.3. Screening and isolation of resistant bacteria to EE2

Subsequently, 100 µL of each dilution was spread on solid plate cultures with mineral salt medium (MSM) a medium without carbon compounds which was composed by 0.5 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.01 mg/L NaCl, 0.2 g/L $MgCl_2.6H_2O$, 0.02 g/L $CaCl_2$, 0.0004 mg/L $ZnSO_4.7H_2O$, 0.0004 g/L $CoCl_2.6H_2O$, 0.0003 mg/L $MnSO_4$, 0.01 mg/L of ethylenediamine tetraacetic acid (EDTA) and 0.0003 mg/L $(NH_4)_6Mo_7O_{24}.4H_2O$), with a nitrogen source of 1 g/L $(NH_4)_2HPO_4$ and agar (15 g/L). The experiments were carried out adding to the solid MSM:

i. 10 g/L glucose, to monitor bacterial growth, given that this compound is the preferred carbon source for most of the common heterotrophic bacteria (Moat *et al.* 2002) as positive control;
ii. 50 mg/L of EE2 (98% purity) purchased from Sigma-Aldrich (Hamburd, Germany) as unique carbon source.

MSM was autoclaved at 120 °C for 45 min and cooled to room temperature before the addition of EE2. The stock solution of 1000 mg/L of EE2 was performed by dissolving

10 mg of drug in 100 µL of methanol and fulfilled with 10 mL of milli-Q water. The required volume of EE2's stock solution to make the drug concentration of 50 mg/L EE2 was added using a PES syringe filter of 0.2 µm pore size from VWR (Leuven, Belgium). The agar plates were placed upside down to prevent condensation on the agar and incubated in the dark at 28ºC for 24, 48 and 144 hours (depending on bacterial growth).

A screening of resistant bacterial isolates colonies was done after 24, 48 and 144 hours to select and isolate resistant bacteria colonies for further tests of drugs biodegradation. The bacterial isolates representing morphologically (pigmentation, shape, size, elevation, margin) different colonies and/or similar morphotypes with ability to grow in the presence of the drugs were picked for further identification and to perform drug biodegradation experiments. Bacterial isolates were stored in 30% (v/v) of glycerol at -20 °C.

Catalase activity was determined using 3% (v/v) hydrogen peroxide solution, a positive reaction is indicated by the production of bubbles. Oxidase activity was evaluated with Microbact oxidase strips (OXOID).

The gram staining protocol was accomplished as described by Gram 1884 and observed on a light microscope (Leica Microsystems).

2.4. Identification of pharmaceutical resistant bacteria

To identify the bacterial isolates able to degrade the drugs under study, biomass was produced for DNA extraction. For biomass production, 100 µL of glycerol culture was poured onto solid PCA growth medium at 23.5 g/L using the spread plate method. Cultures were incubated at 28 °C in the dark for 48 hours. DNA was extracted from each isolate bacterial culture with the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek), following the protocol provided by manufacturer.

The concentration and quality of eluted DNA was determined using a NanoDrop spectrophotometer Thermo Scientific 3300 for further amplification of the DNA extracted through the polymerase chain reaction (PCR).

The 16S rRNA gene was amplified from the DNA samples extracted from the bacterial isolates. For the PCR amplification of the bacterial 16S rRNA gene conserved region the following universal primers were used the 8F (*forward*) 5'-AGAGTTTGATCCTGGCTCAG-3'/ 1492R (*reverse*) 5'-TACCTTGTTACGACTT-3'.

For the reaction mixture, 2 µL of DNA sample, 1 µL of forward primer, 1 µL of reverse primers (8), 2.5 µL of Dream Taq Buffer, 0.1 µL of Dream Taq DNA polymerase and 0. 5 µL dNTPs mixture (NZytech). For a final volume of 25 µL of reaction.

Amplifications were performed on the thermal cycler 2720 (Applied Biosystems) using the following conditions: initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 1 minute (denaturation), 57 °C for 30 seconds (primer annealing) and 72 °C for 2 minutes (elongation), and final elongation at 72 °C for 5 minutes.

The gel electrophoresis was performed using 5 µL of amplified PCR product to which it was added 2 µL of loading dye buffer x6 (NZYtech) containing blue and orange bromophenol dyes G on an agarose gel (1% agarose, Tris- 1% Acetate-EDTA (TAE)). According to the manufacturer's instructions (Invitrogen), 3 µL of DNA safe SYBR (NZYtech) was added to the agarose. 5 µL of 1 kb NZYDNA Ladder VII (NZYtech) was used as the molecular marker to estimate, by comparison, the size of the DNA fragments analyzed. The migration was performed at 100 V for 1 hour. The gel was examined in a UV transilluminator (Biorad Universal Hood II Gel Doc System, UK) for visualization of the DNA fragment bands.

2.4.1. DNA sequencing

The obtained isolates were further identified by DNA of 16S rRNA genes amplification (originating a purified PCR product of 1484 bp). Direct sequencing of the PCR products was performed by the Sanger method. The sequencing reaction was performed using the purified PCR product (the template amount *per* sequencing reaction for amplifications between 1000 and 2000 bp should be about 40 ng of DNA) corresponding to the amplification of the 16S rRNA gene with *primer*s 8F/1492R and the *BigDye Terminator Cycle sequencing* v*3.*1. kit (Applied Biosystems, USA) (with a labeling dye for each of the nucleotides). The POP-7 polymer (Applied Biosystems, USA) was used as separation matrix for performing DNA sequencing and fragment analysis in a Genetic Analysis 3130xl sequencer (Applied Biosystems, USA). The results were obtained by Sequencing Genetic Analysis Software, provided by CCMAR's Molecular Biology platform.

The sequence reads analysis were treated using the BIOedit Sequence Alignment Editor and contig-0 sequences were generated as listed in **Table S6 (**available in the **Annex 5**). Taxonomic identity was established through Blast search of Gene Bank database of NCBI and RDP Classifier (Wang *et al*. 2007).

2.5. EE2 biodegradation assays

Liquid cultures were used to study the biodegradation of the drug by the bacterial isolates. After bacterial isolates identification, the further experiments were carried out in liquid medium using the MSM performed as described above supplemented with a nitrogen source of 1 g/L of $(NH_4)_2HPO_4$ and a carbon source: 10 g/L glucose (positive control) or 13 mg/L of the drug. The concentrations of 20 and 50 mg/L of EE2 were also tested but these bacterial isolates were not resistant to that concentrations in liquid medium. Negative controls only with the drug and in the absence of the bacterial isolates were performed. The bacterial isolate cultures were incubated at 28 °C at 150 rpm in the dark to avoid drug's photodegradation. Sampling was performed after 24 h, 48 h and 168 h. The assays were performed in triplicate.

The bacterial growth was monitored by measuring the optical density (OD) at 600 nm using a Hach-Lange™ DR 2800 UV/visible spectrophotometer (Sköndal, Sweden).

The doubling time for each bacterial isolate growth in cultures spiked with EE2 was calculated during exponential growth, assuming that OD is directly proportional to the number of cells (not always) during the time (T) of the experiment. The growth rate was calculated from two points in the linear part of the curve giving by using $r = \frac{(LN\left[\frac{OD_2}{OD_1}\right])}{(\mathrm{T2}-\mathrm{T1})}$ and the doubling time corresponds to $\frac{LN(2)}{r}$.

2.6. Biodegradation of EE2

2.6.1. Analysis of EE2 by Gas Chromatography-Mass Spectrometry (GC-MS)

To determine the concentration of EE2 samples corresponding to the four isolates were prepared in triplicates. All samples were lyophilized and resuspended in methanol. 50 µL of each resuspended sample were taken in 2 mL glass vials and dried under gentle nitrogen stream. Prior GC-MS analysis a chemical derivatization by silylation was carried out. Derivatization solution was performed with 280 µL of N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), 11.2 mg NH_4I and 16.8 µL beta-mercaptoethanol with MSTFA for GC derivatization, ≥98.5%, Sigma-Aldrich (Switzerland) in a dilution of 1/9. 50 µL of derivatization solution was added to each dried sample into the vials. The vials were sealed, and the solution was heated on a bath at 60 ºC for 30 min. Derivatized samples were then analysed by GC–MS.

The column was a Restek-Rxi® - 5 Sil MS (Crossbond®, similar to 5% diphenyl/95% dimethylpolysiloxane) 30-meter, 0.25 mm ID, 0.25 μm df). Instrument parameters were as follows: Injection volume 1 μL, injector temperature, 250 ºC in mode splitless, oven temperature programming was initial temperature 160 ºC, hold for 2 min, 9 ºC/min to 260 ºC, hold for 5 min and 260 ºC to 300 ºC at 20 ºC/min, hold for 5 min at final temperature, flowrate 1 mL/min, transfer line 250ºC, source 220ºC, carrier gas helium. The calibration curve was performed using standards of EE2 at five different concentrations from 1 to 12 mg/mL.

One-Way ANOVA (Single Factor) tests using Excel Data Analysis Tools were performed to evaluate the differences between the cultures with inoculum in the presence or absence (negative control) of the drug, with a significance level (α) of 0.05.

3. RESULTS AND DISCUSSION

3.1. Identification and classification of bacterial isolates resistant to EE2

Experiments were performed using as inoculum a sludge collected in an oxidation ditch from Faro Northwest WWTP, in MSM solid medium in the presence of glucose (positive control) and 50 mg/L of EE2, as the sole source of carbon source and supplemented with a nitrogen source, in order to select the microorganisms that were resistant and use this drug for its growth. From this experiment it was possible to successfully isolate four bacteria with ability to grow in the presence of EE2 as unique carbon source.

The almost complete 16S rRNA gene sequences of the four strains were determined (1484 bp). The sequence reads obtained by Sanger sequencing were treated using the BIOedit Sequence Alignment Editor and the generated contig-0 sequences (**Table S6** available in the **Annex 5**) were analyzed through a BLAST search of Gene Bank database of NCBI (**Table 7.1**) and by RDP classifier (Wang *et al*. 2007).

Table 7.1 Identification of bacterial isolates that grew in the presence of 50 mg/L EE2 using 16S rRNA gene sequencing by NCBI Blast database

Sample name	From cultures	Specie (NCBI Blast)	Query cover (%)	Identity (%)	Accession nº (NCBI Blast)
1	50 EE2	*Acinetobacter bouvetii* strain DSM 14964	100	98.51	NR_117628.1
2	50 EE2	*Acinetobacter kookii* strain 11-0202	95	97.03	NR_135727.1
3	50 EE2	*Pantoea agglomerans* strain JCM1236	99	87.47	NR_111998.1
4	50 EE2	*Shinella zoogloeoides* strain NBRC 102405	90	97.34	NR_114067.1

The isolates 1 and 2 were identified with high percentage of similarity (>97%) as *Acinetobacter bouvetii* strain DSM 14964 (GenBank accession NR_117628.1) and *Acinetobacter kookii* strain 11-0202 (GenBank accession NR_135727.1), respectively by NCBI BLAST database. Both species belong to phylum "Proteobacteria"; class Gammaproteobacteria; order Pseudomonadales; family Moraxellaceae and genus *Acinetobacter* according to RDP classifier (Wang *et al*. 2007)

Isolate 3 was identified as *Pantoea agglomerans* strain JCM1236 (GenBank accession NR_111998.1) sharing 87.47% of similarity with the known specie and classified as belonging to phylum "Proteobacteria"; class Gammaproteobacteria; order "Enterobacteriales"; family Enterobacteriaceae; unclassified_Enterobacteriaceae by RDP classifier. Isolate 4 shares 97.34% of similarity with *Shinella zoogloeoides* (GenBank accession NR_114067.1), which agree with RPD classification where this isolate belongs to phylum "Proteobacteria"; class Alphaproteobacteria; order Rhizobiales; family Rhizobiaceae and genus *Shinella*.

The morphological and biochemical characteristics of these four bacterial isolates that grew successfully in the presence of EE2 are described in the **Table 7.2**.

Table 7.2 Colony characteristics and biochemical tests (oxidase and catalase) of the bacterial isolates *Acinetobacter bouvetii*, *Acinetobacter kookii*, *Pantoea agglomerans* and *Shinella zoogloeoides*

Isolate	Bacteria	Oxidase	Catalase	Gram staining	Color	Colony characteristics
1	*Acinetobacter bouvetii*	-	+	-	Light beije	Circular, convex, smooth and light opaque
2	*Acinetobacter kookii*	-	+	-	Light yellow	Circular, smooth, opaque with entire margin
3	*Pantoea agglomerans*	+	-	-	Beije-yellow	Smooth, more or less convex with entire margin
4	*Shinella zoogloeoides*	-	-	-	white-yellow	Smooth opaque with irregular margins

'+' = positive reaction, '-'= negative reaction

The morphological and biochemical characteristics obtained for each studied isolate are in accordance with those reported in the literature for the bacteria posteriorly identified (Carr *et al*. 2003; Gavini *et al*. 1989; An *et al*. 2006).

Species from the genus *Acinetobacter*, *Pantoe* and *Shinella* are herein presented for the first time as having ability to degrade EE2. Although they were already reported as having ability to degrade other organic compounds thus being of great interest in WWTP's processes for pollutants removal. For example:

Acinetobacter genus is characterized as phenol-degrading microorganisms which can use it as sole energy and carbon source (Liu *et al*. 2016). Some strains of this genus are known to have a role in the biodegradation of a wide number of different pollutants, such as biphenyl and chlorinated biphenyl, amino acids (aniline), phenol, benzoate, crude oil, ACN, and in the removal of phosphate or heavy metals (Briganti *et al*. 1997; Wenthur, 2016). It has also been reported that certain *Acinetobacter* strains can utilize biphenyls including chlorinated biphenyls. Furthermore, some *Acinetobacter* isolated from mixed cultures showed the ability to completely mineralize monohalogenated biphenyls (Shields *et al.* 1985). Some strains of this genus were also employed in the degradation of lignin and amino acids (Abdel-el-haleem, 2013; Buchan *et al*. 2001; Kahng *et al*., 2002).

Pantoea genus was found to have a potential contribution in the removal of polycyclic aromatic hydrocarbons. It was demonstrated that *Pantoea* show a high biodegradation ability for polycyclic aromatic hydrocarbons and presented high tolerance to strict environments such as resistance to different ranges of pH, salt, and different polycyclic aromatic hydrocarbons concentration. During the biotransformation process, mechanisms of adsorption/desorption can play a significant role in the bioavailability of polycyclic aromatic hydrocarbons (Cai *et al*. 2011; Seo *et al.* 2009; Zhao *et al*., 2018).

***Shinella* genus** have been reported to degrade the derivatives of 1H-1,2,4-triazole, such as triazophos, tebuconazole, ipconazole, propiconazole and nicotine (Qiu *et al*. 2014). *Shinella* sp. strain HZN7 strain was the first nicotine-degrading bacterium to be isolated from the genus *Shinella*, metabolizing nicotine into non-toxic compounds. It is also shown degradation of the toxic pollutants, 4-aminobenzenesulfonate (Qiu *et al*. 2016).

3.2. EE2 biodegradation experiments

To test the ability of these isolates to grow in the presence of EE2 and degrade it, liquid cultures were performed in the presence of 13 mg/L EE2 as sole carbon and $(NH_4)_2HPO_4$ as nitrogen sources.

The four bacterial isolates demonstrated the capacity to grow in the presence of 13 mg/L of EE2, which was verified by an increase in optical density measured at 600 nm (OD_{600nm}) in comparison to the negative control over the course of the assay. The observed optical densities for *Acinetobacter bouvetii, Acinetobacter kookii* and *Pantoea agglomerans* were of 0.484 ± 0.003, 0.237 ± 0.004, 0.318 ± 0.002 after 48 h, respectively and 0.553 ± 0.008, 0.343 ± 0.006, 0.328 ± 0.001 after 168 h, respectively. The growth of *Shinella zoogloeoides* showed the lowest OD values of 0.02 ± 0.001 and 0.035 ± 0.001, after 48 h and 168 h incubation, respectively. During the degradation experiment of 13 mg/L EE2, the doubling time or generation time for both *Acinetobacter bouvetii* and *Acinetobacter kookii* was of approximately 20 h, for *Pantoea agglomerans* was 22 h while for *Shinella zoogloeoides* was approximately 24 h, hence these bacteria divide in every 20 h, 22 h and 24 h, respectively.

A positive control in the presence of 10 mg/L glucose was performed, in order to check the bacterial growth in presence of their preferential carbon source, which was expected to be glucose. As expected, all isolates grew in presence of glucose, with maximum OD_{600nm} values achieved with *Pantoea agglomerans* (≈ 0.650) and lowest with

Acinetobacter bouvetii (≈ 0.050). On the other hand, in presence of EE2, *Acinetobacter bouvetii* achieved considerably better growth with OD_{600nm} values of 0.553 ± 0.008. This clearly shows that *Acinetobacter bouvetii* will preferentially use EE2 over glucose as carbon source. The doubling time for both *Acinetobacter bouvetii, Acinetobacter kookii* was approximately 16 h, while for *Pantoea agglomerans* and *Shinella zoogloeoides* were 23 h and 20 h, respectively, meaning that they consume EE2 at a similar rate as glucose, making them able to switch and adapt to carbon sources other than glucose, which is a characteristic of gram-negative bacteria (Buffing *et al*. 2018). Thus, if *Acinetobacter bouvetii*, was grown in a mixture of two carbon sources (EE2 and glucose), these bacteria would first deplete EE2 in MSM and then switch to glucose. As for *Pantoea agglomerans*, it would start first with glucose and EE2 would be used after all the glucose in media is spent. However, this was not confirmed in practice in this study, but it should be considered for the future.

The removal of EE2 by the obtained isolated bacteria *Acinetobacter bouvetii, Acinetobacter kookii, Pantoea agglomerans* and *Shinella zoogloeoides* after 168 h is represented in **Figure7.2**.

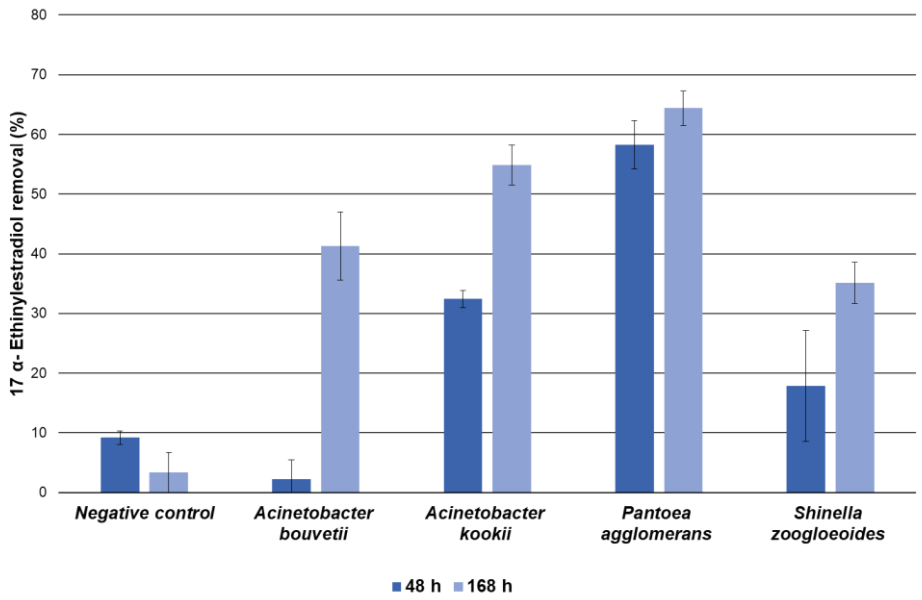

Figure 7.2 The removal percentages of 13 mg/L of EE2 by the obtained isolated bacteria *Acinetobacter bouvetii, Acinetobacter kookii, Pantoea agglomerans* and *Shinella zoogloeoides*, after 48 and 168 h incubation at 150 rpm in the dark

The **Figure 7.2** showed that *Acinetobacter bouvetii, Acinetobacter kookii, Pantoea agglomerans* and *Shinella zoogloeoides* removed 2 ± 3%, 32 ± 1%, 58 ± 4% and 18 ±

9% of 13 mg/L EE2, respectively, after 48 h of incubation. After 168 h incubation the removal efficiencies of EE2 by *Acinetobacter bouvetii, Acinetobacter kookii, Pantoea agglomerans* and *Shinella zoogloeoides* were 41 ± 6%, 55 ± 3%, 64 ± 3% and 35 ± 3%, respectively. The highest percentage of removal of the drug was obtained by *Pantoea agglomerans* (64 ± 3%) and the lowest by *Shinella zoogloeoides* (35 ± 3%) (**Figure 7.2**).

In the negative control (cultures with drug without bacteria) the removals of EE2 were of 9 ± 1% and 3 ± 3%, after 48 and 168 h of incubation (**Figure 7.2**). All the obtained bacterial isolates showed a significant effect ($P<0.05$) on removing EE2 when compared with the negative control.

Although *Pantoea agglomerans* presented the highest removal of EE2 it did not displayed the highest optical density. Several works showed that drug degradation was not dependent on bacterial concentration, hence the low OD_{600nm} values for highest percentage of EE2 removal. Several explanations can be given to this fact:

i) the bacterium tends to get resistance during the assay and subsequent contact with the drug starting to slowly use EE2 as carbon source or could co-metabolically degrade the drug;

ii) Fioravante *et al*. (2012) reported that EE2 was likely accumulated within the bacterial cells (bioaccumulation) rather than adsorbed in the surface of the cells (biosorption) although existing also this last possibility.

As already herein referred it has been documented that strains from the genus *Pantoea* show biodegradation activity on various chemical pollutants especially of soil and water, including polycyclic aromatic hydrocarbons, petroleum hydrocarbons and toxic metals (Cai *et al*. 2011; Seo *et al*. 2009; Zhao *et al*. 2018; McGuinness and Dowling, 2009). The high EE2 degradation by the *Pantoea agglomerans* used in this study compared to the others was prospective since this genus is known to be capable of degrading organic compounds with aromatic structures and phenolic moieties like EE2.

Pauwels and colleagues (2008) reported that *Acinetobacter* sp. was not able to metabolize EE2, but it was co-metabolized in the presence of E1, E2 and E3.

3.3. Identification of the metabolites produced during the degradation of EE2

The metabolites formed during the experiment with subsequent degradation of EE2 were analyzed by GC-MS. The trimethylsilyl (TMS) derivatives of the detected

secondary metabolites were similar for the four bacterial isolates; however, the compounds which are herein considered are the ones obtained with the bacterial isolate that presented the highest degradation (*Pantoea agglomerans*). The GC-MS of the TMS derivatives of metabolic products and its molecular structures produced during the degradation of EE2 are presented in **Table S7** and **Figure S5** of **Annex 5**.

As shown in the **Table S7** of **Annex 5**, other degradation compounds than the ones produced along with EE2 described in the literature were herein detected and assumed as metabolites.

Several studies reported degradation of natural and synthetic estrogens and investigated the metabolism of E2, EE2, estrone and estriol (E3) by activated sludge, night-soil composting microorganisms and bacterial isolates and proved the degradation of the natural hormones E2, E1 and E3 but not the synthetic steroid EE2 (Shi *et al*. 2004; Yu *et al*. 2013; Pauwels *et al*. 2008).

Thus, research into identification of strains of microorganisms that can break down estrogens, giving special attention to synthetic EE2, with the possible application to wastewater treatment became mandatory.

Shi *et al*. (2002) have isolated from cowshed samples an EE2-degrading microorganism *Fusarium proliferatum* which uses EE2 as the sole source of carbon. They have reported the existence of EE2-degradative products, which may be more polar compounds that have a phenolic group, however these substances stood to be identified.

However, can be predicted that the metabolism of the E2 and E3 natural steroid estrogens may have a similar degradation pathway as E1 and probably of EE2 (Koh *et al*. 2008).

Lee and Liu (2002) studied E2 degradation by mixed bacteria of an activated sludge in the very early stages and proposed a cleavage and oxidation of E1 at the D-ring producing a lactone structure. In addition, Kurisu *et al*. (2010) proposed that E2 suffer oxidation at the aromatic A-ring producing 4-hydroxyestrone with further attack of the saturated B-ring, yielding metabolites keto-estradiol and keto-estrone. Li *et al*. (2012) further hypothesized that E1 was converted to tyrosine following cleavage of a saturated ring. Haiyan *et al*. (2007) proposed an EE2 degradation pathway for *Sphingobacterium* sp. JCR5 in which EE2 is oxidized to E1 followed by cleavage of the B ring and the A ring. Intermediate metabolites are the unsaturated acids 2-hydroxy-2,4-dienevaleric acid and 2-hydroxy-2,4-diene-1,6-dioic acid which are ultimately mineralized to CO_2 and water. Haiyan *et al*. (2007) also reported a testosterone-degrading bacterium

Comamonas testosteroni TA441 that performed an analogous pathway, where a latter metabolite had a different cleavage position of 3-hydroxy-4,5-9,10-disecoestrane-1(10),2-diene-5,9,17-trione-4-oic acid. Thus, the detection of EE2 metabolites suggest that the drug's degradation pathways may involve oxidation of the ethynyl group or hydroxylation of the aromatic ring.

O'Grady *et al*. (2009) detected a metabolite from EE2 transformation by *R. erythropolis* with a proposed chemical structure involving oxidation of the ethynyl group to an acetic acid moiety. EE2 metabolites involving hydroxylation of the A ring, producing 2-hydroxy-EE2, as well as cleavage of the A ring have been reported for nitrifying activated sludge (Yi and Harper, 2007; Yi *et al*. 2006; Lust 2014). Also, ammonia oxidizing bacteria have been reported to co-metabolize EE2 when oxidizing ammonia. This was associated to a specificity of the ammonium mono-oxygenase enzyme (Vader *et al*. 2000; Joss *et al*. 2004; Yi and Harper, 2007).

Although E1 was not herein identified as EE2 metabolite during its degradation as described in the literature, since this compound may be quickly converted as for example referred by Weber *et al*. 2005 that E1 was no longer detectable after 72 h.

Other possible intermediates were detected, such as acid mannonic acid 1,4-lactone which are gamma lactone structures, thus being a lactone at D ring as proposed by Lee and Liu (2002). However, this lactone structure herein detected was already cleaved apart from the C ring of EE2. 2,3-dihydroxybenzoic acid was identified, this compound seems to be already cleaved apart from the B ring, while molecules which were detected like (Z)-aconitic acid, 2-pentenedioic acid an unsaturated acid and 2-butenedioic acid that may have already suffered meta-cleavage of the A ring. This last one, 2-butenedioic acid or fumaric acid is a dicarboxylic acid and intermediate metabolite in the TCA cycle. Lee and Liu (2002) believed that these intermediary metabolites may suffer ring cleavage and can be further degraded by entering the TCA or citric acid cycle. Other intermediate metabolites may be produced as prosta-5,13-dien-1-oic acid that was formed likely due to the opening of the A, B and C rings, that may also be cleaved and supposedly enter the TCA cycle. Coombe *et al*. (1966) detected a tetrahydroquinoline as one of the degradative products when estrone was exposed to *Nocardia sp.* (E 110), whereas in the present study was detected a 4-hydroxy-2-quinolinecarboxylic acid.

Malonate the ionized form of malonic acid was detected, this compound is a potent inhibitor of cellular respiration - inhibits succinate oxidation due to competition with malate (Green and Greenamyre, 1995; Zeevalk *et al*. 1995).

Other compounds such as acetic acid, octadecanoic acid, myristic acid were detected and may be metabolites of other bacterial pathways which were occurring simultaneously.

To the best of our knowledge *Acinetobacter bouvetii*, *Acinetobacter kookii*, *Pantoea agglomerans* and *Shinella zoogloeoides* are identified for the first time as EE2 biodegraders. Likely using these species as a community, the degradation of EE2 could be complete due to synergetic effects between the bacteria.

Thus, culturing these strains in bulk and introducing them into the biological reactors of wastewater treatment plants could potentially reduce the concentrations and estrogenic activities of estrogens released into the environment (Koh *et al*. 2008).

4. CONCLUSIONS

- Bacteria with ability to grow in the presence of 50 mg/L of EE2 was isolated from a sludge collected in the oxidation ditch of Faro's Northwest WWTP, being these, *Acinetobacter bouvetii*, *Acinetobacter kookii*, *Pantoea agglomerans* and *Shinella zoogloeoides* species.
- The results showed that *Acinetobacter bouvetii*, *Acinetobacter kookii*, *Pantoea agglomerans* and *Shinella zoogloeoides*, *Pantoea agglomerans* and *Shinella zoogloeoides* were able to degrade 47 ± 4%, 55 ± 3%, 64 ± 4% and 35 ± 4%, respectively of 13 mg/L EE2 after 168 h at 28 ºC.
- Several possible intermediates were detected, such as mannonic acid 1,4-lactone which is a gamma lactone structure, 2,3-dihydroxybenzoic acid, (Z)-aconitic acid, 2-pentenedioic acid an unsaturated acid and 2-butenedioic acid that may have already suffered meta-cleavage of the A ring, were also detected. Also, 2-butenedioic acid (fumaric acid) which is a dicarboxylic acid and an intermediate metabolite in the TCA cycle was identified.

To the best of our knowledge the bacterial species herein presented are indicated for the first time as having a role in EE2 and also the detected compounds resulting from the metabolic pathways were different from the already described in the literature. This research shows the importance of the involved bacteria for the effectiveness of the removal of EE2 in a WWTP.

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CHAPTER 8

Assembly, start-up and optimization attempt of an aerobic granular sludge SBR system

A modified version of this chapter would be published as:

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ABSTRACT

Nowadays with the increasing pollution by arising chemicals such as the pharmaceuticals, it is of a great concern to find new and innovative wastewater treatment solutions aiming the detoxification of aquatic environments. The construction of an aerobic granular system plant to modernize wastewater treatment for the cities of Faro and Olhão by Águas do Algarve is ongoing. The company is interested in research regarding granule ability to degrade pharmaceuticals detected in wastewater influent. In this work, two aerobic granule sequencing batch reactors were constructed and several attempts to optimize the system were performed. The operating conditions and efficiency of the reactors in the removal of COD (acetate as carbon source), ammonium, nitrate, nitrite and phosphate were investigated aiming the degradation of ibuprofen. The reactors were operated at laboratory scale using different cycles (anaerobic/aerobic and aerobic) and several alterations were implemented, attempting to improve the system, mainly in the reactor feeding. The best results were obtained with the addition of injector pumps. In this operation mode, COD reduction was easily achieved in the aerobic cycle, although ammonium and nitrate were partially removed in this phase. A partial denitrification was obtained in the anaerobic phase. The maximum IBU removal reached about 82% after 6 hours and of 20% after 10 hours. This occurred probably due to the association of a major adsorption mechanism associated to biodegradation. Also, older granules could display less ability to biodegrade but adsorption may still occurrs. However, and due to difficulties related to the achievement of optimal system conditions, such as, feeding, aeration, shear stress, homogenization during the feeding and in the anaerobic step (poor homogenation possibly leads to stratification), a lot remains to be studied and the optimization of the system is still ongoing.

1. INTRODUCTION

In the last decades, with the huge advances of science and technology, there are more and more scientific studies reporting the presence of new compounds, known by emerging pollutants, in wastewater and aquatic environments (Deblonde *et al*. 2011; Sui *et al*. 2015; Mendoza *et al*. 2016; Wang *et al*. 2016). The US Environmental Protection Agency (US EPA) defines emerging pollutants as new, unregulated chemical compounds whose impact on the environment and human health is poorly understood (Deblonde *et al*. 2011). These compounds may be metals, pesticides, phthalates, polycyclic aromatic hydrocarbons, endocrine disruptors, cytostatic drugs, antibiotics, food additives, stimulants and illicit drugs, among others (Deblonde *et al*. 2011; Mendoza *et al*. 2016; Brumovský *et al*. 2017).

The production and use of large amounts of emerging pollutants inevitably results in the release of these compounds into the environment, usually through sewage and WWTP discharges (Sui *et al*. 2015). The biodegradation and behaviour of these compounds in aquatic systems remain largely unknown, resulting in an underestimation of their true importance in terms of toxic effects on the environment. These compounds can also have an impact on wastewater treatment processes by increasing or decreasing their efficiency when removing contaminants from sewage or industries (Gavrilescu *et al*. 2015).

WWTP are usually unable to completely remove these pollutants from municipal waters, thus becoming a risk to public and environmental health. These contaminants are characterized as “pseudo-persistent”, as they are not completely removed and are constantly being released into the environment. Although some of these pollutants are degradable, their metabolites, sometimes more toxic than the parent compound, are also not effectively removed, becoming persistent in aquatic ecosystems (Ebele *et al*. 2017). Bioaccumulation of these pollutants may occur, affecting the organisms in the ecosystems where they have been detected even at residual concentrations, because many of these compounds remain biologically active (Ebele *et al*. 2017).

Thus, the necessity to implement new wastewater treatment technologies that can be able to remove COD, nitrogen and phosphorus and all other toxic compounds from waters.

The conventional activated sludge system is a technology widely used in sewage and wastewater treatments. In activated sludge systems, bacterial communities usually develop in the form of suspended "flocks" (activated sludge) in the wastewater to be

treated. These conventional systems consist in 2 physically separated tanks; the first is an aeration tank where biological reactions such as organic carbon removal and nitrification take place and a second one is a sedimentation tank where activated sludge is separated from the treated water by a flocculation process (Nancharaiah and Reddy, 2018).

This system presents some disadvantages such as: (i) requires two process tanks, one for aeration and another for "flocks" sedimentation that in turn need (ii) a large area for its implementation, (iii) the aeration process and recycling stream demand for a high energy and operating cost, (iv) a low biomass concentration in the aeration tank, (v) a poor sedimentation capacity that can deteriorate effluent quality, leading to a treatment efficiency drop (Nancharaiah and Kiran Kumar Reddy, 2018).

The relatively recent and promising aerobic granular sludge system (AGS) is used as an alternative to the conventional activated sludge system. Compared to the activated sludge process, the granular system has several advantages such as: (i) it is constituted by denser and stronger structures of microbial aggregates (macroscale, in the order of millimetres), (ii) a smaller area is required (80% less) for the process implementation, since effective recycle flow rate can be much smaller than conventional WWTP (10 to 15 times). In addition, the (iii) granules have a high biomass concentration and retention and (iv) faster settling properties, (v) good biosorption properties, (vi) ability to withstand shock loads to chemical toxicity because of that strong and complex granule structures, also (vi) presenting a much higher efficiency and much lower energy cost compared to activated sludge systems. Also, (vii) cycles can be adjusted to influent characteristics, (viii) weather conditions, (ix) actual sludge conversion rates, (x) desired effluent conditions and the (xi) granular sludge selection pressure (Tay *et al*. 2002; Pronk *et al*. 2015; Nancharaiah and Reddy, 2018; Wilén *et al*. 2018).

AGS offers an interesting alternative due to its excellent physical characteristics, being its biofilm composed of self-immobilized microbial communities (Beun *et al*. 1999). These granules have a compact structure and are biologically efficient, constituted a large diversity of microorganisms (Adav *et al*. 2008). The stratification of the conversion processes and the redox zones within the granule give rise to layers with different aerobic and anaerobic/anoxic microenvironments that allow simultaneous removal of carbon, nitrogen and phosphorus (De Kreuk and Van Loosdrecht, 2005; Zhang *et al.* 2016; Nancharaiah and Reddy, 2018). The representation of a granular stratified structure is shown in **Figure 8.1**.

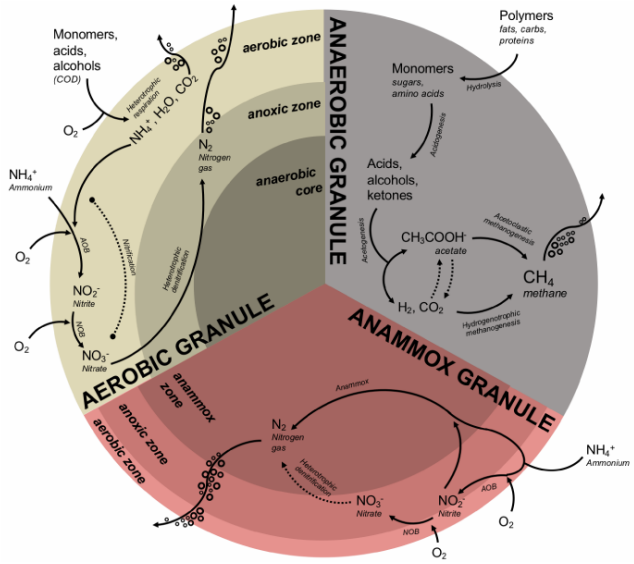


Figure 8.1 Combined schematic illustrating zones of activity, and biochemical conversion, characteristics of anaerobic, aerobic and anammox granules (adapted from Trego *et al.* 2019)

Granules are commposed by three zones: anaerobic, aerobic and anammox (**Figure 8.1**). In an outer layer, during the aeration (presence of oxygen) the growth and metabolic processes of polyphosphate accumulating organisms (PAO) which remove phosphorus and glycogen accumulating organisms (GAO) are favoured. Simultaneously, there are the ocurrence of (i) nitrification with the oxidation of nitrite to nitrate by nitrite oxidizing bacteria in the aerobic zone and (ii) denitrification with the reduction of nitrate to nitrite and subsequently to nitrogen by denitrifiers in the annoxic zone (**Figure 8.1**) (De Kreuk and Van Loosdrecht, 2005; Nancharaiah and Reddy, 2018).

The stability and efficiency is directly dependent on the size of the granule (optimum diameter of 2-4 mm), to which dissolved oxygen saturation levels are critical. Early studies of aerobic granular systems employed mainly aerobic cycles (Beun *et al*. 2002). However, with the performed optimizations for granule stability, efficiency and effluent production respecting to EU standards, it was determined that the anaerobic/aerobic cycle is best suited. Operational differences lie in the longer length of influent feeding and shorter aeration period in the anaerobic/aerobic cycle *versus* the aerobic cycle (Adav *et al*. 2008; Beun *et al*. 2002; Ni *et al*. 2009; Lee *et al*. 2010; Lochmatter and Holliger, 2014; Awang and Shaaban, 2016).

These granules are usually grown in an aerated sequencing batch reactor (SBR) where short settling periods are applied in order to allow the selection of the larger granules (greater aggregation) and washing the ones with lower settling ability (Nancharaiah and

Reddy, 2018). This system works in 4 phases: influent inlet, aeration period, sedimentation period and removal of the treated effluent (**Figure 8.2**).

The **Figure 8.2** illustrates an example of system operation in sequencing batch reactors (SBRs) with a four-phase operational cycle.

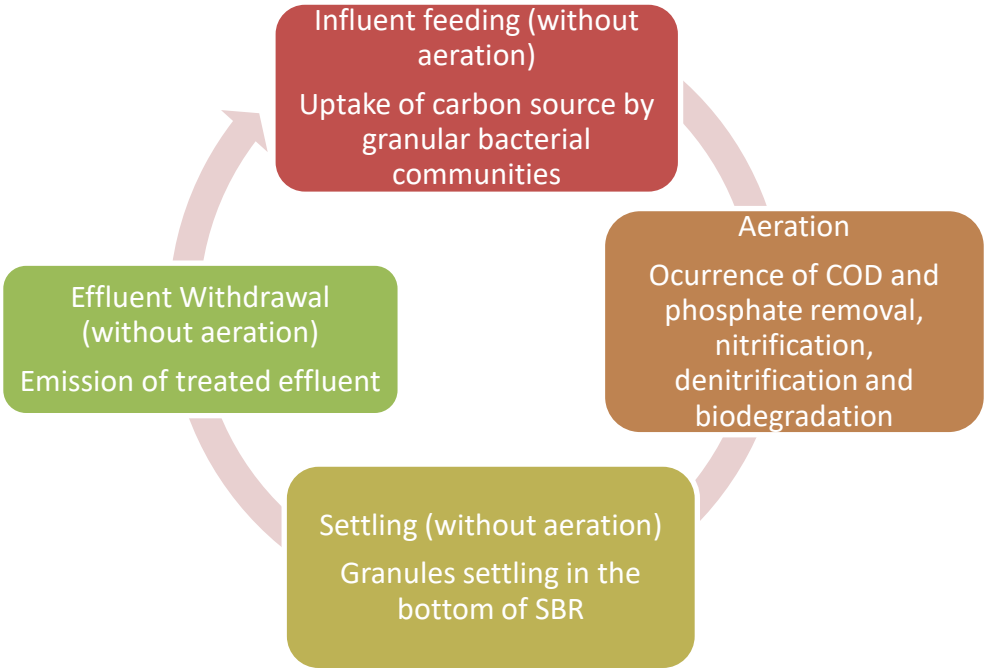


Figure 8.2 Operation cycle of aerobic SBRs (adapted from Adav *et al*. 2008)

The aeration period in the SBR consists of two phases: in the first one the substrate degradation occurs and in the second one this is no longer available, this lack of substrate results in granule stabilization (Adav *et al*. 2008).

Due to these characteristics, this system of granules began to be used for the treatment of wastewater with high amounts of organic matter, nitrogen, phosphorous, among other toxic and xenobiotic substances (Adav *et al*. 2008).

SBRs can be started using activated sludge or already formed granules as inoculum. Several parameters can be monitored and/or measured to assess SBR efficiency and granules applicability with the desired research objective (Beun *et al*. 2002; Ni *et al*. 2009; Lee *et al*. 2010; Lochmatter and Holliger, 2014; Awang and Shaaban, 2016).

Several studies demonstrated the granule's biodegradation potential, provided by easy adaptation to the use of different carbon sources, influent composition and operational cycle and their high resistance to toxic compounds (Jiang *et al*. 2002; Moy *et al*. 2002; Tay *et al*. 2002; Liu *et al*. 2005; Schwarzenbeck *et al*. 2005; Tay *et al*. 2005; Adav *et al*.

2009). These characteristics are what confer the granules with a great potential for application to pharmaceuticals detoxification (Jiang *et al*. 2002; Moy *et al*. 2002; Tay *et al*. 2002; Liu *et al*. 2005; Schwarzenbeck *et al*. 2005; Tay *et al*. 2005; Adav *et al*. 2009). Amorim *et al*. (2014) reported that drugs such as ofloxacin, norfloxacin and ciprofloxacin were degraded in an AGS.

Ibuprofen is a NSAID pharmaceutical with analgesic (reduce pain), antipyretic (reduce fever) and anti-inflammatory properties. This drug is conjugated in the liver and excreted in urine as water-soluble glucuronides and sulphates. Several studies may understimate the real concentration of the incoming water, thus the screening disregarded the conjugated NSAID and only detected the free fraction of NSAID (Remberger *et al*. 2009). Ibuprofen, in terms of toxicity, has not shown harmful effects on the growth of some fungi and bacteria (Ferrando-Climent *et al*. 2012). However, Schnell *et al*. (2009) demonstrated that IBU when combined with other drugs in the environment may inhibit the growth of human embryonic cells, yet cytotoxicity of this drug occurs at higher concentrations than those found in the environment (Schnell *et al*. 2009). Wang and Gardinali (2013) demonstrated that IBU is bioaccumulated by *Gambusia holbrooki* fish (mosquito fish) with a bioaccumulation factor of 28. The drug slowly enters the fish organism and is subsequently eliminated after a half-life of 32 h.

Target compounds were detected in most wastewater and river water samples of KwaZulu-Natal Province in South Africa, with IBU being the most frequently detected pharmaceutical. Maximum concentrations detected in river water for naproxen, ibuprofen, and diclofenac were 6.84, 19.2, and 9.69 µg/L, respectively (Madikizela and Chimuka, 2017). Reematsma (2006) reported 0.1-1 µg/L of IBU in European WWTP effluents, while Remberger and colleagues (2009) identified 2.6-3.5 µg/L of the drug in Hemlunda (Sweden) WWTP effluent. In the Netherlands this compound can be found in influent WWTPs ranging from 3 to 39 µg/L (Kujawa-Roeleveld and Schuman, 2006). In Portugal, the concentrations of IBU found in the influent WWTPs are 3, 3.9, 5.3 and 9.8 µg/L in spring, summer, autumn and winter, respectively and in the effluent are 0.16, 0.3, 1.5, 1.8 µg/L in spring, summer, autumn and winter, respectively (Pereira *et al*. 2015; Pereira *et al*. 2016). In Spain the concentrations of IBU and its metabolites were found in a WWTP's influent at 13.74, 5.8, 38.4 and 94.0 µg/L of IBU, 1-hydroxyibuprofen (1-OH IBU), ibuprofen carboxylic acid (CBX IBU) and 2-OH IBU, respectively and in the effluent at 1.9, 1.4, 10.7 and 5.9 µg/L of IBU, 1-OH IBU, CBX IBU and 2-OH IBU, respectively (Ferrando-Climent *et al*. 2012). Despite several IBU

degradation studies in the treatment processes and on its fate in the environment have been performed, there is still a lack of information regarding the presence and toxicity of the drug and its metabolites in the aquatic environment. These metabolites are produced not only by human metabolism but also by the activity of microorganisms present in natural resources such as waters, soils and sediments as in WWTP sludge (Ferrando-Climent *et al*. 2012). The persistence of ibuprofen and its secondary metabolite 2-OH IBU is low, with a dissipation time between 3.1 to 7 days (Ebele *et al*. 2017). Higher concentrations of IBU are measured in colder countries, colder seasons and in WWTPs close to densely populated areas (Wang *et al*. 2011; Pereira *et al*. 2016). Ibuprofen removal is approximately 90% in WWTPs (Ferrando-Climent *et al*. 2012). This high removal is due to treatment using activated sludge in which there is a combination of biodegradation (sludge bacterial communities) and sludge adsorption processes (Ferrando-Climent *et al*. 2012). Although this value may vary according to the initial concentration of ibuprofen in the influent; the higher the concentration in the influent lower is the removal of the drug by the bacterial community (Londoño and Peñuela, 2015).

In the present study a granular sludge sequencing batch reactor operating system was constructed (**Figure 8.3**) and several attempts to its optimization were performed. The SBRs were started with aerobic granules (2 g/L, total suspended solids) from the Águas de Portugal Nereda® Frielas WWTP in Lisbon, Portugal.

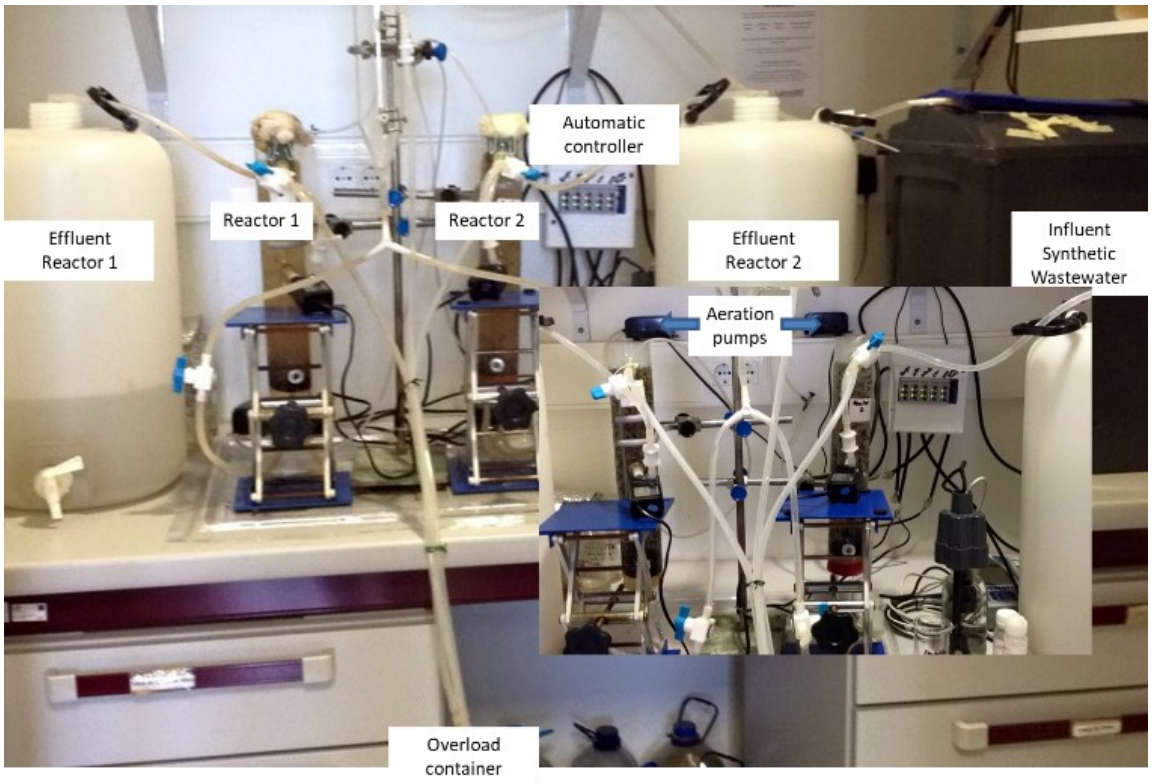


Figure 8.3 Photo of the granular sludge SBR operating system implemented

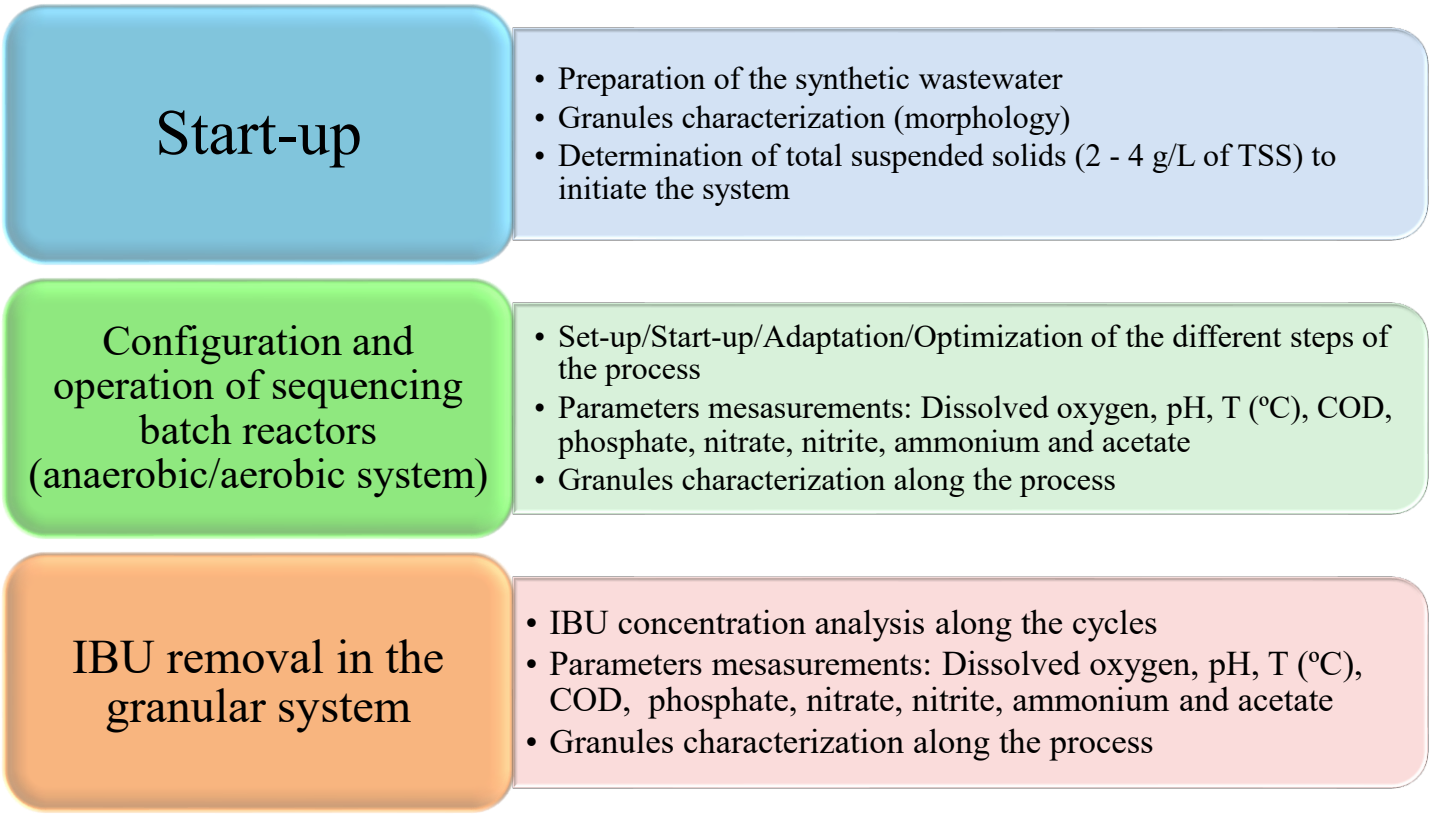
The conditions, parameters and kinetics of reactions in the granular system (nitrogen, nitrate, phosphate, total solids, size of granules) were investigated for further addition of the pharmaceuticals. Dissolved oxygen (mg/L) and pH was monitored. COD (mgO_2/L), phosphorus (mg PO_4^{3-}/L), ammonium-nitrogen (NH_4-N), nitrates (NO_3^--N) and nitrites (NO_2^--N) were determined. Sludge volume index was determined by measuring the time taken to settle to the bottom of the reactor after cessation of aeration.

2. MATERIALS AND METHODS

Schematic diagram of the granular system experiment, which was divided into 3 parts:

1- Start-up: Granules Inoculation (2-4 g/L Total Suspended Solids) in synthetic wastewater with acetate as carbon source;
2- Process optimization in aerobic/anaerobic system: system maintenance and water quality parameters analysis;

3- Biodegradation assays of IBU in the granular system.



2.1. Synthetic wastewater preparation

Synthetic wastewater was prepared using two media, named as (A) and (B) in order to simulate typical characteristics of wastewater. Media A was composed of sodium acetate (63 mM), magnesium sulphate heptahydrate (3.6mM) and potassium chloride (4.7 mM). Media B was composed of ammonium chloride (35.4 mM), dibasic potassium phosphate (4.2 mM), monobasic potassium phosphate (2.1 mM) and 10 mL/L trace element solution (micronutrients). The trace element solution was prepared using ethylenediaminetetraacetic acid (50.0 g/L), zinc sulphate heptahydrate (22.0 g/L), calcium chloride, calcium chloride (5.54 g/L), manganese chloride tetrahydrate (5.06 g/L), iron sulphate heptahydrate (4.99 g/L), ammonium heptamolybdate tetrahydrate (1.10 g/L), copper sulphate pentahydrate (1.57 g/L), cobalt chloride hexahydrate (1.61 g/L). The pH was adjusted to pH 7 using potassium hydroxide. For a final volume of 1.6 L was added 15 mL of solution A and 15 mL of solution B mixed with 1.3 L of tap water.

2.2. Granules characterization - Inoculum for sequencing batch reactor (SBR) start-up

Aerobic granules (10.3 g/L total suspended solids) generously supplied by the Águas de Portugal Nereda® municipal wastewater treatment plant in Frielas, Lisbon, was used as inoculum (2 g/L, total suspended solids) for start-up of the SBRs. The diameter of granules was measured using SteREO Lumar Flourescence Stereomicroscope (Carl Zeiss, Göttingen, Germany) with amplification of 13× using AxioVision software.

2.3. Determination of Total Suspended Solids (TSS)

The total suspended solids of the granules were measured in order to determine the volume to be placed in each reactor for start-up, using the laboratory protocol of the Wastewater Treatment discipline of the Integrated master's in environmental engineering, Faculty of Science and Technology, University of Algarve. The referred protocol was as following: an empty Whatman filter (110 mm, pore size) was placed in the desiccator and posteriorly weighed and placed in a Buchner funnel attached to a filtration apparatus. 100 mL of the granule suspension was measured into a beaker and gently shaken for proper homogenization. 3 mL was removed from the beaker using a pipette with a cut-off tip; the vacuum system was turned on and the sample was evenly applied to the empty filter. The filter was washed three times (5 mL distilled water) under vacuum, removed using tweezers to a watch glass and dried at 110 ºC for 1 h. The dried filter was placed in the desiccator for approximately 20 min to balance the temperature; The filter is weighed on an analytical balance. The value of total suspended solids was calculated as:

$$\text{TSS} = \frac{\text{final weight of filter with granules (mg)} - \text{weight of empty filter (mg)}}{Operation\ volume\ added\ to\ filter\ (L)}$$

2.4. Configuration and operation of SBRs

2.4.1. SBR set-up

The experiment was started with two SBRs, that were constructed using glass cylinders as reactors with a total height of 35 cm and internal diameter of 6 cm. The working volume was of 763 mL, calculated using the following equation $V = \pi r^2 h$, taking into consideration the diameter (d) of 5.8 cm and height (h) of 27 cm as the maximum

influent height. The filled volume was of 395 mL obtained using the same equation but with h of 14 cm. The height was measured by a ruler. Synthetic wastewater (influent) was introduced via two tubes at the bottom of the SBRs. Effluent was withdrawn at a height of 13 cm from the bottom. Both systems were operated in cycles using an automatic timer (Carlo Gavazzi DMB51 multifunction timer) to control the pumps for influent addition, aeration and effluent withdrawal. Aeration was performed at the bottom of the reactor using a tube connected to a fine bubble aerator. The aeration has been stopped during influent addition, settling and effluent withdrawal. The flow rate for reactor 1 was 194 L/h, given by the sum of pump flow rates of 144 L/h and 50 L/h, while the reactor 2 was of 90 L/h.

In a first optimization attempt the reactors were operated for 2 months at room temperature (23 ± 2 ºC) in successive cycles of 168 min (2.8 h, 8.6 cycles daily), according to the equations:

- *Total Operation Time* = 1 min (settling) + 3 min (effluent withdrawal) + 2 min (filling) + 162 min (total aeration) = 168 min (2.8 h)
- *Number of cycles* = $24\ h/2.8\ h$ = 8.6 cycles.

The hydraulic retention time (HRT) was calculated by:

$$\text{HRT} = \text{total cycle length} \Big/ \left(\frac{Volume\ withdrawn}{\text{Total working volume}}\right) = 2.8\ \text{h}/(395/763)\text{mL} \times\ 1/24\ h$$

= 5.39 h or 0.225 days.

The hydraulic loading was calculated as:

$$\text{Hydraulic loading rate} = \text{Total volume of influent added daily} \ / \text{surface area of reactor},$$

where ($3.14 \times d^2/4$) is the surface area of reactor.

Thus, hydraulic loading rate = 3397 mL/cycles per day/($3.14 \times 6^2/4$)=120 cm^3/cm^2 per day or 0.120 m^3/m^2 per day.

Reactor 1 was operated in an aerobic cycle 156 while reactor 2 was operated in the typical anaerobic/aerobic cycle of aerobic granular sludge reactors and wastewater treatment plants (*e.g.* Beun, Van Loosdrecht and Heijnen, 2002; De Kreuk, 2006; Ni *et al*. 2009; Lee *et al*. 2010; Khursheed *et al*. 2012; Morales *et al*. 2013; Pronk *et al*. 2015). The SBR reactor scheme is presented in **Figure 8.4**.

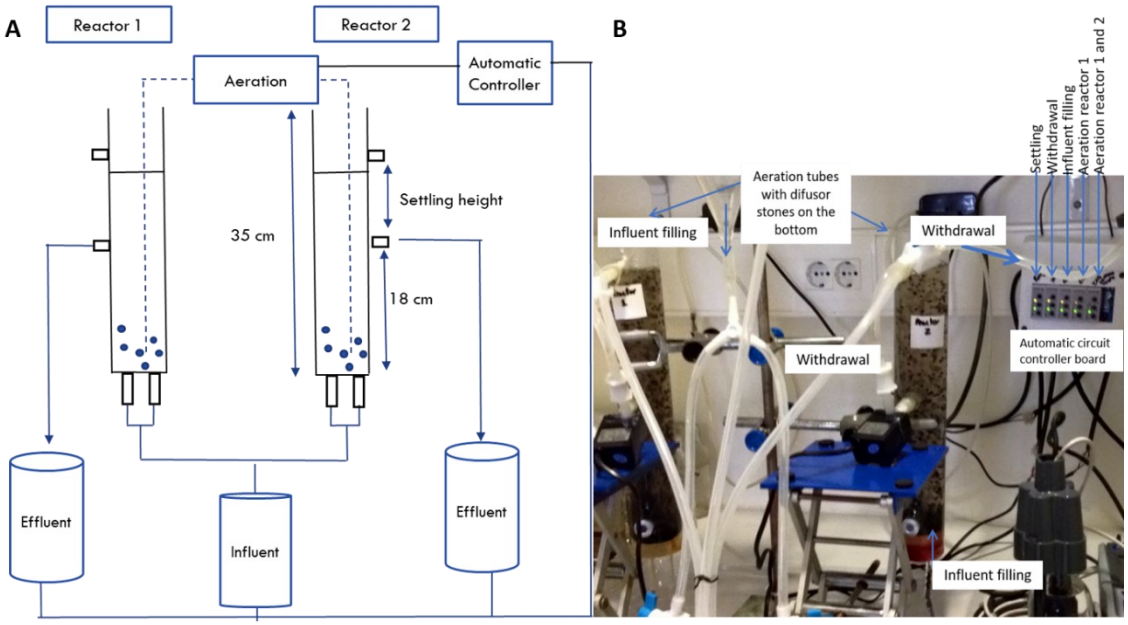


Figure 8.4 Schematic representation (a) and photo (b) of the granular sludge SBR operating system implemented

The initial phases and respective duration are presented in **Table 8.1**.

Table 8.1 Initial phases and respective duration of a complete cycle of the granular sludge SBRs

Phases	Reactor 1 (aerobic)	Reactor 2 (anaerobic)
	Time (min)	
Influent Feeding (no aeration/anaerobic) a. Filling from halfway point b. Additional contact time	2	2 42
Aeration	162	120
Settling (no aeration)	1	
Effluent Withdrawal (no aeration)	3	
Total Cycle Length	**168 (≈ 3 hr)**	**168 (≈ 3 hr)**

An additional contact time of 42 min was programmed for reactor 2 to prolong contact of influent with the granules due to the short feeding time. In each cycle, 395 mL was withdrawn, which accounted for 52% of the reactor volume (calculated using height of the withdrawal point of 13 cm).

2.4.2. Characterization of the influent and effluent

The different parameters COD, phosphorus (as phosphate), nitrate nitrogen, nitrite nitrogen and ammonia were measured using LANGE commercially available kits supplied by HACH (Carnaxide, Portugal). The kit specifications are given in **Table 8.2**.

Table 8.2 Parameters and kits used for characterization of SBR influent and effluent

Parameters	Kit	Concentration range (mg/L)*	Measurement wavelength (λ) - nm
Ammonium (NH_4^+-N)	LCK 304	0.015 - 2.0	694
COD	LCK 514	100 - 2000	605
Nitrate (NO_3^--N)	Powder pillows, cadmium reduction method – 8039	0.3 - 30.0	500
Phosphate (PO_4^{3-})	USEPA Phosver3® (ascorbic acid) method 8048	0.02 - 2.50	880
Nitrite (NO_2^--N)	USEPA Diazotization Method 8507	0.002 - 0.300	507

*Indicated by manufacturer

Dissolved oxygen (mg O_2/L) and pH was monitored by offline instrumentation but not controlled. COD (mg O_2/L), phosphate (mg PO_4^{3-} /L), nitrate (mg NO_3^--N/L), nitrite (NO_2^--N) and ammonium (mg NH_4^+-N/L) were determined on days 14, 17, 20, 21 and 27 using the commercial kits already indicated. Sludge volume index was determined by measuring the time taken to settle to the bottom of the reactor after cessation of aeration.

2.5. Ibuprofen and acetate quantification by HPLC

The SBRs were dosed with IBU twice, first with 2 mg/L on day 85 of operation and with 7 mg/L on day 128. Samples (T0 to T6) were withdrawn 7 times from both reactors respectively, during one cycle at times corresponding to 0, 3, 6, 7, 8, 9 and 10 h, to determine the presence and concentration of IBU. The first and last samples

corresponded respectively to introduction of influent and effluent withdrawal. Samples were filtered using sterile syringe filters (0.2 µm pore size) to remove biomass and bacteria. Controls were prepared as IBU was introduced to the reactors, by spiking synthetic wastewater and milli-Q water respectively in the same dosage. The controls could stand for the length of the same cycle during which sampling was performed, to assess if degradation occurred by other mechanisms.

The quantification of acetate (carbon source) and IBU in influent and effluent was performed using the Advanced Scientific Instrument KNAUER HPLC coupled with a Smartline UV 2600 Smartline Manager 5000 detector (Berlin, Germany). The software ClarityChrom® was used to monitorize and signal integration.

(i) Acetate was detected at wavelength 210 nm, at room temperature and a flow rate of 0.5 mL/min. The mobile phase was 0.0025 M sulphuric acid using a Rezex RFQ-Fast Acid H+ (8%) column supplied by Phenomenex (Torrance, CA, USA).

(ii) IBU was separated by an Xbridge® reverse phase C18 column (hybrid particle, 250 mm length, 4.6 mm i.d, 5.0 µm particle size) attached to a Xbridge C18 guard column (20 mm length, 4.6 mm i.d, 5.0 µm particle size) (Waters Corporation, Milford MA, USA).

For the biodegradation analysis of IBU, HPLC was used by gradient method in which the mobile phase was composed of ACN and water, both acidified with formic acid (0.01%). The gradient started with 25% water eluent in the mobile phase and ended with 80% of it in 15 min of run. The flow was 1.5 mL/min at room temperature. The injection volume was 20 µL. The wavelength for detecting the drugs under study was at 230 nm.

3. RESULTS/DISCUSSION

3.1. Inoculum for SBR Start-up

The SBRs were started with aerobic granules (2 g/L, total suspended solids) from the Águas de Portugal Nereda® Frielas WWTP in Lisbon, Portugal. The TSS of the supply was measured at 10.3 g/L. The size of the granules ranged from 2 to 5 mm with the average size of 3 ± 1.2 mm (**Figure 8.5**).

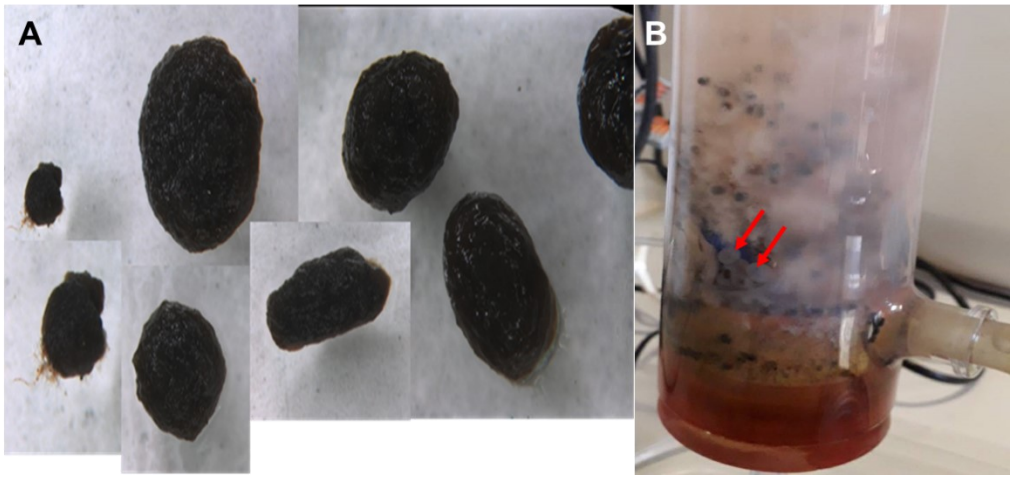


Figure 8.5 Photos of granular sludge by SteREO Lumar.V12 Fluorescence Stereomicroscope, amplification of 13X using AxioVision 4.8.2 software (A); formation of white granules indicated by the red arrows, during the SRB system operation (B)

Twenty granules were measured and the obtained sizes ranging from 2 to 5 mm, with an average size of 3 ± 1.22 mm. The characteristics of the obtained granules agree with the finding of Toh *et al*. (2003) (**Figure 8.5**). They separated a sludge sample into a range of size fractions and found that granule size varied from <0.5–4 mm, with the most part between 1 and 3 mm in diameter. The aggregates are usually described as round or spherical (Dangcong *et al*. 1999; Tay *et al*. 2001), but also irregular shapes have been reported (Jang *et al*. 2003). Size distribution of granules is one of the most important characteristics, associated with the density of the aggregates, since this determines the settleability and retention in anaerobic digestion systems (Bellouti *et al*. 1997; Jeison and Chamy, 1998). Several studies described the effect of hydrodynamics on granule size (Arcand *et al*. 1994; Chang and Lin, 2004), but others (*e.g*. Batstone and Keller, 2001) found feed (wastewater) to be a stronger influence (Trego *et al.* 2019). For example, Tay *et al*. (2001) compared granulation under glucose and acetate feeds and concluded that the substrate type was determinant for granule size, with glucose producing bigger granules. Díaz *et al*. (2006) separated granules based on colour: black, grey, or brown. They observed that varied granule structures corresponded to different colours, where black granules were small and compact, grey granules were more layered, and brown granules were less compact and more porous. Díaz *et al*. (2006) suggested that the larger, brown granules corresponded to older aggregates, and, further, they hypothesized that the different colour 'types' were at different development stages. However, in the present work it seems to be an acceptable hypothesis since the older

aggregates presented, in fact, brown colour. During the experiment white granules appeared, these ones are associated with the granules in formation (Wilén *et al*. 2018). Normally, aerobic granules are smaller than anaerobic granules, ranging from 0.2–2.0 mm in diameter, sometimes reaching 3 mm (Cydzik-Kwiatkowska *et al*. 2016; Jang *et al*. 2003; Tay *et al*. 2001; Zheng and Yu, 2007; Trego *et al.* 2019).

3.2. Start-up - Performance of aerobic granule SBRs

Two aerobic granule SBRs were operated in different conditions as described in Material and Methods section 2.4. The SBRs were constructed by adapting material available in the laboratory. The glass tubes used as reactors were of smaller dimensions than those used in the reported aerobic granule studies with pharmaceuticals (Hu *et al*. 2012; Si *et al*. 2013; Amorim *et al.* 2014; Moreira *et al*. 2015; Amorim *et al*. 2016; Mihciokur and Oguz, 2016). The SBRs were dosed with IBU at two concentrations (2 and 7 mg/L) in order to assess the ability and efficiency of aerobic granules to biodegrade IBU. Initially, identical pumps for both reactors were used, however due to a failure, it was necessary to replace the pump of reactor 2. The hydraulic retention time was 5.4 h and influent loading rate was 0.120 m^3/m^2 per day (equations in the Material and Methods section 2.4). The measured pH ranged from 7.4 to 8.4. The dissolved oxygen was 8.26 mg/L. SBR performance was evaluated by COD, phosphate (phosphorous), nitrate and ammonium removal (**Figure 8.6**) before and after adding the IBU after 21 days of working.

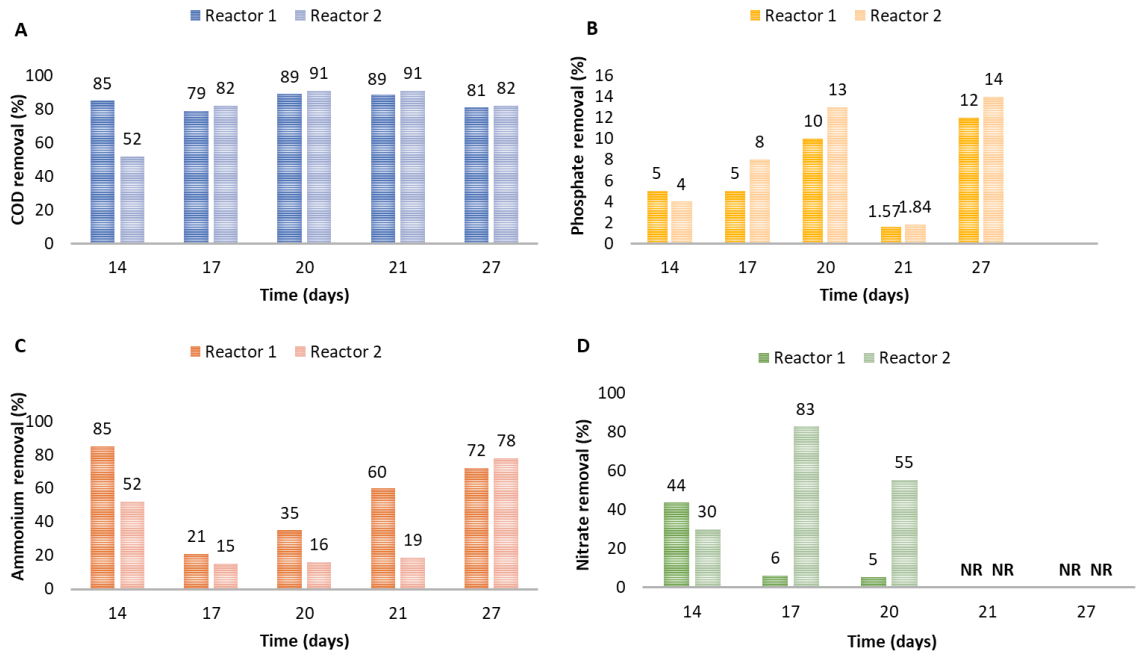


Figure 8.6 COD (A), phosphate (B), ammonium (C) and nitrate (D) removal percentages in the influent and effluent, during 27 days of SBR operation.
NR-no removal

COD removal was initially higher in reactor 1. However, over time there was a balance between them, with an increase in COD removal in reactor 2. After 17 days of operation both reactors presented a high COD removal of approximately 90% with a slight decrease to about 80% after 27 days. It was observed that COD values decreased below regulatory limit of 125 mg O_2/L in 42 min of operation for both systems. Also, it should be noted that IBU was added to reactors in the days 21 and 27 and that the presence of drug seemed to not affect the COD removal. Under typical SBR operating conditions (anaerobic/aerobic reactor 2), the highest consumption of COD by the microorganisms occurred in the aerobic phase (**Figure 8.6A**).

Early aerobic granule SBRs had a short feeding period, as the one initially applied to the SBRs in this study, until research proved that longer feeding periods were required to maintain granule stability at full-scale operation (De Kreuk, 2006). Phosphorous (measured as PO_4^{3-}) removal, which was reported to occur during the aerobic stage, was initially slightly higher in reactor 1, but the removal in reactor 2 improved, surpassing the reactor 1 after 17 days of operation. However, the maximum removal observed was of 14%, after 27 days of operating in the presence of IBU. This removal eventually took place when the reactor 2 was under aeration (**Figure 8.6B**). Low phosphate removal is a consequence of competition between polyphosphate and glycogen accumulating

organisms for COD uptake (Beun, 2001; Amorim *et al*. 2016). Ammonium removal was higher in reactor 1 until day 27 when the removal in both reactors were balanced. IBU appeared to have no negative effect in COD removal since the profiles observed were similar to previous results obtained before the addition of the drug and also the results were similar to other aerobic granule SBR studies with pharmaceuticals (*e.g.* Hu *et al*. 2012; Si *et al*. 2013; Amorim *et al.* 2014; Moreira *et al*. 2015; Amorim *et al*. 2016; Mihciokur and Oguz, 2016). A comparison of nitrate concentration in the influent and effluent of both reactors is shown in **Figure 8.6D**. Nitrate effluent concentration was initially lower in reactor 1 at day 14. Subsequently, reactor 2 showed the best removal of nitrate until days 21 and 27, when nitrate concentration was higher in the effluent of both reactors in comparison to the influent. This indicates the suppression of denitrification, causing nitrogen accumulation that could be due to the accumulative effects of the constant high dissolved oxygen in the reactors, which reduces granule nitrification ability, or due to the addition of IBU to the SBRs, or both. Shi *et al*. (2013) and Amorim *et al*. (2016) observed this effect in aerobic granule SBRs dosed with chiral pharmaceuticals and tetracycline, respectively. The addition of IBU on day 21, also appeared to significantly reduce phosphorus removal. However, reactor performance recovered to normal levels, even with the addition of IBU on day 27, indicating granule adjustment to IBU. Ammonium and nitrate removal were usually good in previous studies where dissolved oxygen was monitored but not controlled. However, larger reactors were used than those employed in this study, which could have a role in reducing this effect. Confirmation of the effect of IBU in producing these effects is required by comparison to SBRs operated in the absence of IBU. After 17 days, the granule layer increased by 61% (**Figure 8.7**), with a sludge volume index (SVI), method used to describe the settling characteristics of sludge in the aeration tank in activated sludge processes, of 25 seconds.

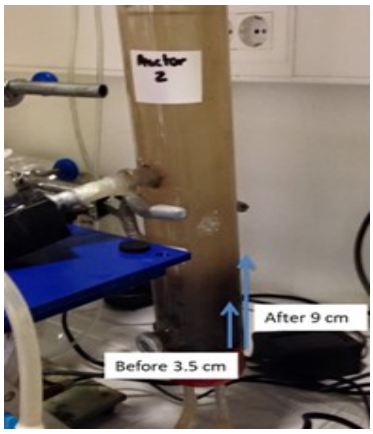


Figure 8.7 Representative photo of the granular sludge growth in the reactor 2 (aerobic/anaerobic) system, after 17 days SBR operation

However, after one month of operation, filamentous bulking and slime in the aerobic granules was observed, indicating possible nutrient deficiency. Fungal growth was also observed in the influent containers. The acetate concentration in the freshly prepared synthetic wastewater, influent and effluent respectively was analysed by HPLC. The results confirmed that acetate concentration of the influent introduced to the SBRs was low, therefore the granules were not receiving the carbon load needed to maintain stability and form. The acetate degradation was likely occurring inside the influent reservoir by opportunistic bacteria or fungi since the influent was stored in between cycles. The operations of both SBRs were halted until a solution could be implemented to avoid this problem.

3.3. Reactor optimization

In this second attempt the granules used were stored at 4 °C for about 8 months (time of waiting for the infusion pumps) before being reinoculated in the reactor with synthetic wastewater. The granular system was operational for 128 days at room temperature (T = 23 ± 1 ºC), with an average influent pH of 7.55 and effluent 8.14.

As it was intended to optimize the operating conditions of SBR and wastewater treatment in the presence of pharmaceuticals, namely IBU, the phases and cycles were tailored regarding the needs as the study progressed. The Start-up was performed using the mode that previously demonstrated the best operating conditions, therefore, the aerobic/anaerobic system (working conditions are described in the **Table 8.1** in the Material and Methods section). A syringe infusion pump was installed, in order to optimize the system, due to the fast acetate consuming in the influent container leading

to a lack of nutrient feeding. This enables the addition of solution A separated from solution B in enough quantity for a complete cycle without causing the early depletion of acetate (**Figure 8.8**).

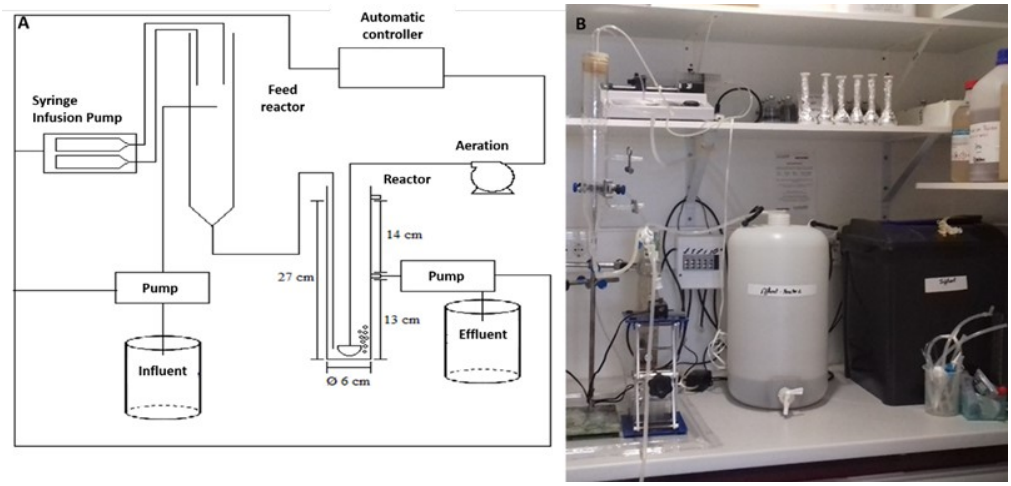


Figure 8.8 Schematic representation (A) and photo (B) of the granular SBR system with implemented modifications

The contact time was subsequently increased from 42 min to 2 h (granules settled-improving anoxic/anaerobic processes) with 42 min aeration. However, later the reactor started to operate in 4 h cycles, in order to optimize COD removal.

COD, ammonium (NH_4^+-N), nitrate (NO_3^--N), phosphate (PO_4^{3-}-P) and nitrite (NO_2^--N) concentrations were analysed in the influent and effluent during the "new" start-up of the SBR that was operating during 36 days with complete cycles of 2 hours and 42 minutes (**Figure 8.9**).

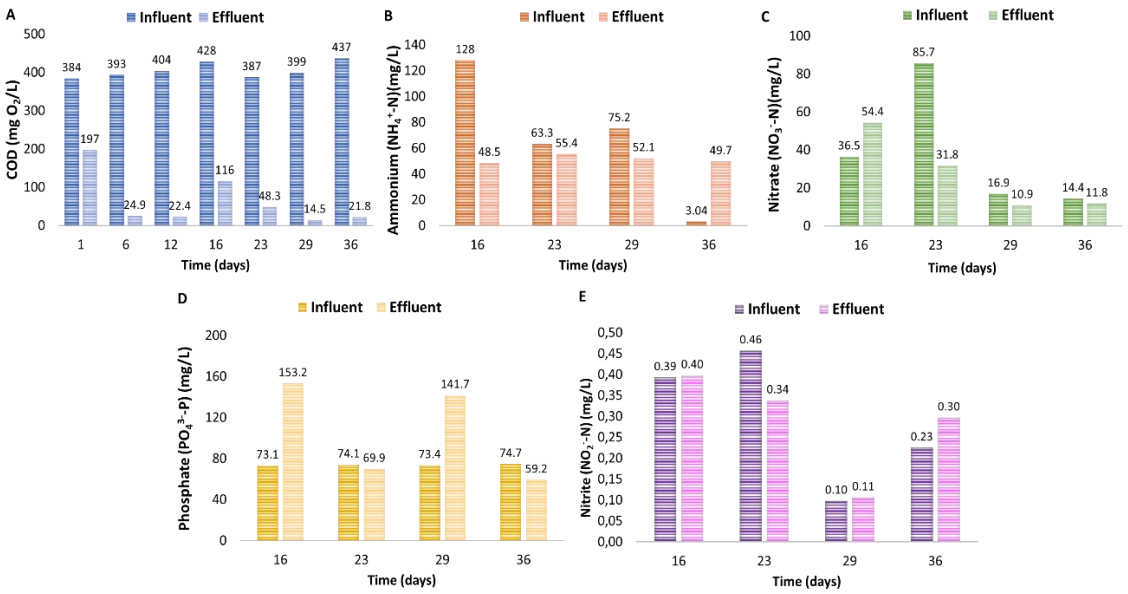


Figure 8.9 Graphical representation of COD (A), ammonium (B), nitrate (C), phosphate (D) and nitrite (E) concentrations (mg/L) in the influent and effluent during the start-up of the SBR (complete cycle of 2 h 42 min)

The results shown that after 24 hours of operation COD decreased in 49%. After 6 days of operation the COD was almost completely removed (94%). In the day 16 a decrease in COD removal (73%) was observed, which may be due an error in sample treatment. Ammonium, nitrate, nitrite and phosphate were also monitored from the day 16 to day 36 (**Figure 8.9**). The maximum removal obtained for ammonium was 62%, after 16 days operation. However, this percentage of removal decreased for 17% and 31% after 23 and 29 days of operation. The ammonium value of the day 36 is likely wrong since the concentration of this compound in the influent was higher than the obtained, an error occurred or during the sample collection or during the analysis (**Figure 8.9A**). Nitrate and nitrite concentrations in the influent and effluent are shown in **Figure 8.9C and 8.9E**, respectively, but it was not possible to achieve complete nitrification and denitrification during the cycle. Nitrate started to be removed after 23 days of operation with 63% of removal. The nitrate concentrations measured in the influent decreased and the removal percentages decreased for 36% and 18%, after 29 and 36 days, respectively. As already mentioned, nitrification occurred in the aerobic phase, thus probably 42 min were not enough time, or the existing bacterial population did not belong to the main responsible for ammonia and nitrate removal. For example, Wei *et al*. (2018) indicated that *Nitrosomonas* affiliated to the ammonia oxidizing bacteria (AOB) was the predominant group with a proportion of 24.1% in the partial nitrification system. The highest removal of nitrite of 26% also occurred in the day 23. Wang and colleagues

(2018) observed that denitrification efficiency was reduced due to the accumulation of nitrate, although the removal of COD remained stable with good efficiency. Also, Wei and colleagues (2018) reported simultaneous free ammonia and free nitrous acid that played major inhibitory roles on the activity of nitrite oxidizing bacteria (NOB). It is also known that denitrifiers and (PAOs) are located in the inner layers of the granules (Winkler *et al*. 2013; Xavier *et al*. 2007).

The phosphate concentrations in the influent and effluent are shown in **Figure 8.9D**. The maximum removal of 21% was obtained after 36 days of operation. It is known that an alternation of anaerobic and aerobic conditions enriches or selects the microbial strains synthesizing polyphosphate (PAOs), that take up phosphate from wastewater at the aerobic stage, while GAOs consume external carbon source at anaerobic stage, but at aerobic stage they do not uptake phosphate from environment and there is no poly-P accumulation occurring (Lin *et al*. 2015). However, after 16 and 29 days of operation it was observed that the phosphate concentration in the effluent surpassed the one of influent, accumulating phosphate. The low phosphate removal or its accumulation may happen due to several factors such as:

(i) as already mentioned, that can be due to competition between polyphosphate and glycogen accumulating organisms for COD uptake (Beun, 2001; Amorim *et al*. 2016). Relative abundance of PAOs over GAOs may be increased by a relative lower dissolved oxygen levels (1.5 to 3 mg/L) at the aerobic stage (Lin *et al*. 2015) or *vice-versa*;

(ii) PAOs population of granules can be older and in minor quantity, thus not possessing the expected performance;

(iii) the existence of predatory bacteria, bacteriophages (virus) and protists (Johnke *et al*. 2014), that has been reported to have relevante implications in the process performance. “Bacteriophages have been reported to display 10 to 100 times higher diversities than bacteria in aquatic ecosystems and to be responsible up to 71% of the bacterial mortality” (Johnke *et al*. 2014). Barr *et al*. (2010) associated an unexpected drop in phosphate-removal performance with bacteriophages infection of the key phosphate accumulating bacterium in a laboratory-scale SBR for enhanced biological phosphorous removal. Predatory bacteria feeding on other microbial cells have been found in a broad range of environments (Martin, 2002). The presence of predatory bacteria in aerobic granules and its persistence during granulation has been reported (*e.g.* Li *et al*. 2014; Szabó *et al*. 2014 Weissbrodt *et al*. 2014; Wilén *et al*. 2018). It is believed that they have an important influence on microbial community structure and

dynamics. Not only phosphate removal is affected, Dolinšek *et al*. (2013) also found evidence of predation of *Nitrospira* sp. by *Micavibrio*-like bacteria that can have a direct impact on the nitrification process.

After 41 days of operation of the granular system, samples were also taken after 42 minutes, corresponding to the end of the anaerobic phase (**Figure 8.10**).

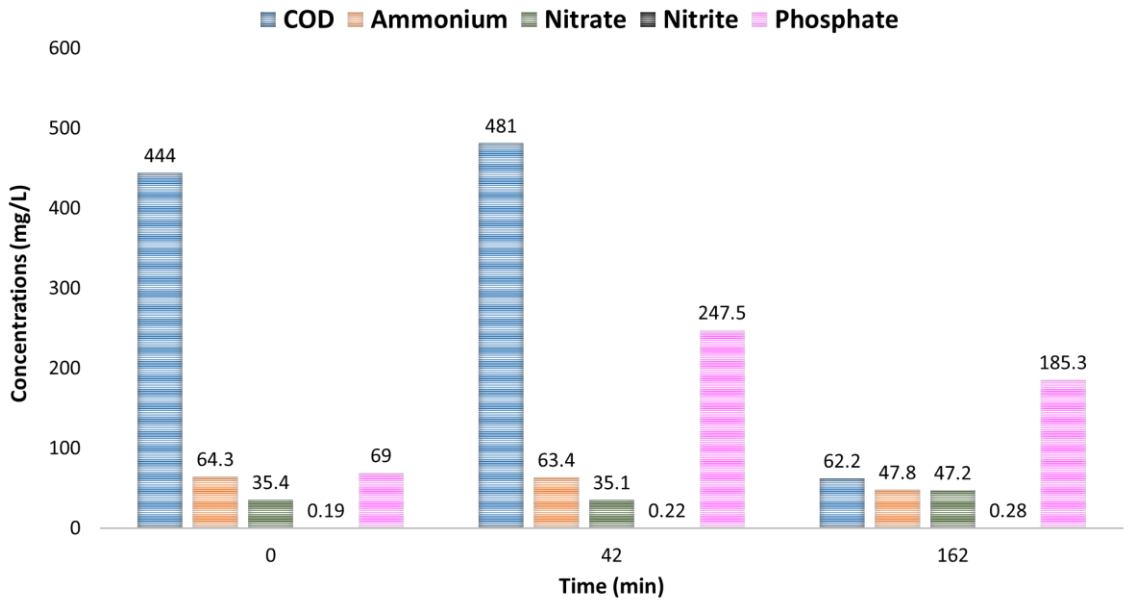


Figure 8.10 Measurement of all parameters of a complete cycle, after 41 days of operation, in influent (0 minutes), at the end of contact time (after 42 minutes) and in the effluent (162 minutes). Complete cycle duration of 2 h and 42 minutes

COD was not removed after 42 min of contact time, thus the bacterial community present in the anaerobic phase of this granular system was not able to degrade the organic material (**Figure 8.10**). Therefore, in the aerobic phase COD and ammonium were removed in 86% and 26%, respectively, while nitrate, nitrite and phosphate were accumulated.

COD was supposed to be removed during the contact time, which did not occur. Therefore, contact time corresponding to the anaerobic/anoxic phase was increased to 1 h and then to 2 h, 3 h and 6 h in order to check COD removal. During 23 days of SBR operation, COD was analysed in the influent, in the final of contact time and effluent as shown in **Figure 8.11**.

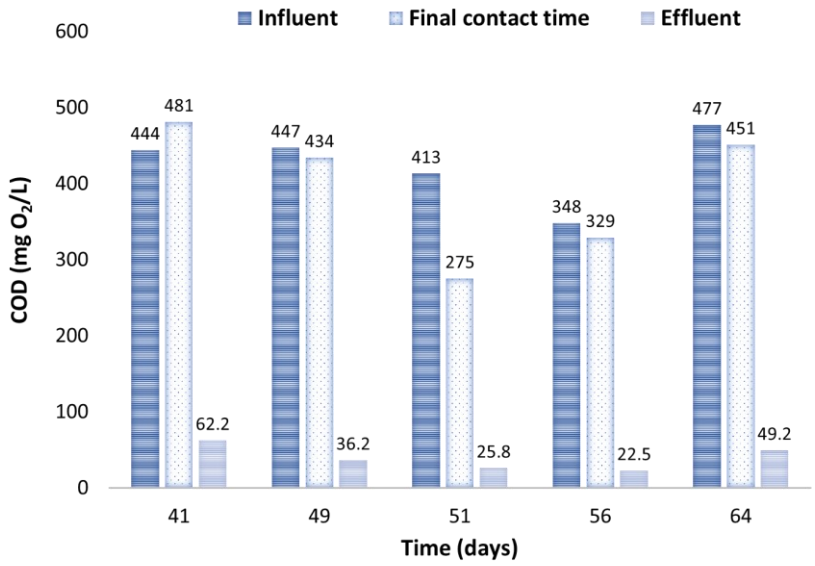


Figure 8.11 COD concentrations (mg O_2/L) in the influent, final contact time and effluent with increasing contact times in order to check its removal by granular bacterial community; day 41 - 42 minutes of contact time (complete cycle - 2h and 42 minutes); day 49 – 1 h contact time (complete cycle of 3 h); day 51 – 2 h contact time (complete cycle – 4 h); day 56 – 3 h contact time (complete cycle of 5 h); day 64 - 6 h of contact time (complete cycle of 8 h)

As shown in **Figure 8.11** the higher COD removal measured in the end of contact time was of 33%. In the remaining days it was observed a slight removal of COD with an exception, after 42 min of contact time COD surpass the influent concentration. However, for all the tested conditions, few minutes after aeration COD removal began and was very high, with a removal percentage ranging from 83 to 94%.

COD measures the amount of organic compounds in water; therefore, its removal is directly related to the consumption of the carbon source (acetate). Thus, it was checked whether it was degraded during this contact time. These results can be seen in **Figure 8.12**, where it was stablished a comparison between COD and acetate removal over the 8 hours cycle of day 69.

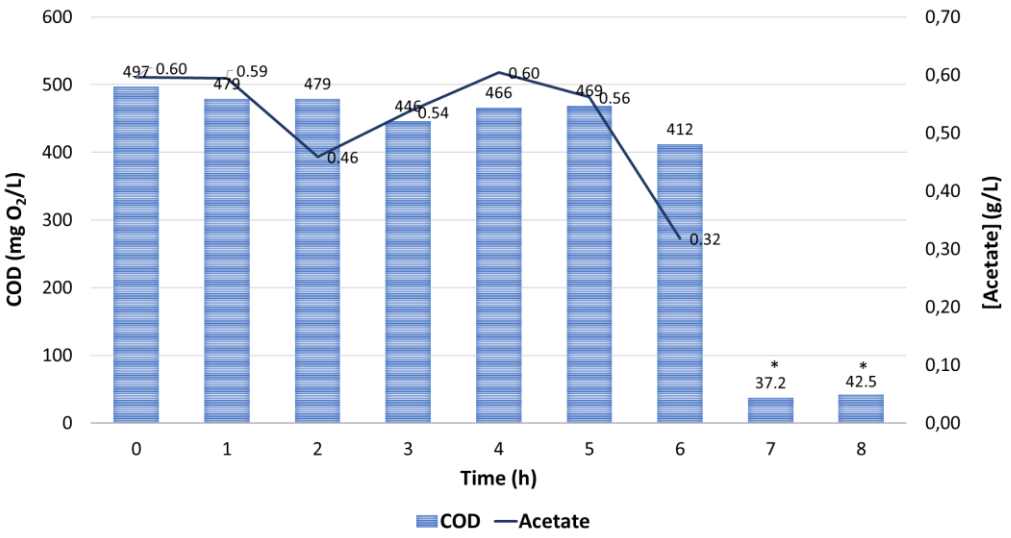


Figure 8.12 COD (mg O_2/L) and acetate (carbon source) (g/L) concentrations after 69 of SBR operating. 8-hour cycle (Contact time during the first 6 h and the remaining 2 h corresponding to aeration period). *below the limit of detection

The results shown that neither COD nor acetate were degraded during the first 6 hours of contact time (**Figure 8.12**). COD decreased only after aeration began and the same was observed for acetate, as it was expected. After 7 and 8 hours of the cycle, it was found that the concentration of acetate was already below the limit of detection of 0.05 g/L **(**the acetate peak was not detected by the HPLC system), revealing its consumption by the bacterial community. It was also verified that after 30 min of aeration the concentration of COD decreased to the desired values. This information can be confirmed by the experiments performed after 77 days of operating, when the cycle was of 8 hours, with 6 hours of contact time without aeration and the 2 last hours corresponding to the aeration period. After 6 h of contact time, a COD concentration of 524 mg O_2/L was observed and after 30 min of aeration at 6 h 30 minutes, this value decreased to 151 mg O_2/L, about 3 times lower than the previous value. After 7 hours the value decreased for 33.6 mg O_2/L COD.

Devlin *et al*. (2017) performed experiments on SRB, where they tested various types of synthetic wastewater with different initial COD concentrations and low hydrodynamic forces during aeration and found that the higher the COD concentration in water, the higher the probability of heterotrophic communities to growth, and therefore, the lower is the efficiency of the system. Devlin *et al*. (2017) also describes that the anaerobic feeding acted as a poor buffer preventing carbon from passing to the aerobic zone, yet this concentration is enough to maintain the heterotrophic microorganisms active.

As the hydrodynamic force of aeration was not sufficient for these communities to compact and form spherical shapes, they formed deformed and poorly compacted filamentous agglomerates (Devlin *et al*. 2017).

3.4. Addition of IBU

Although the system optimization has not been reached, after 85 days of operation, IBU was added in order to test the performance of SBR in the presence of this drug and the ability of the system for its degradation (**Figure 8.13**).

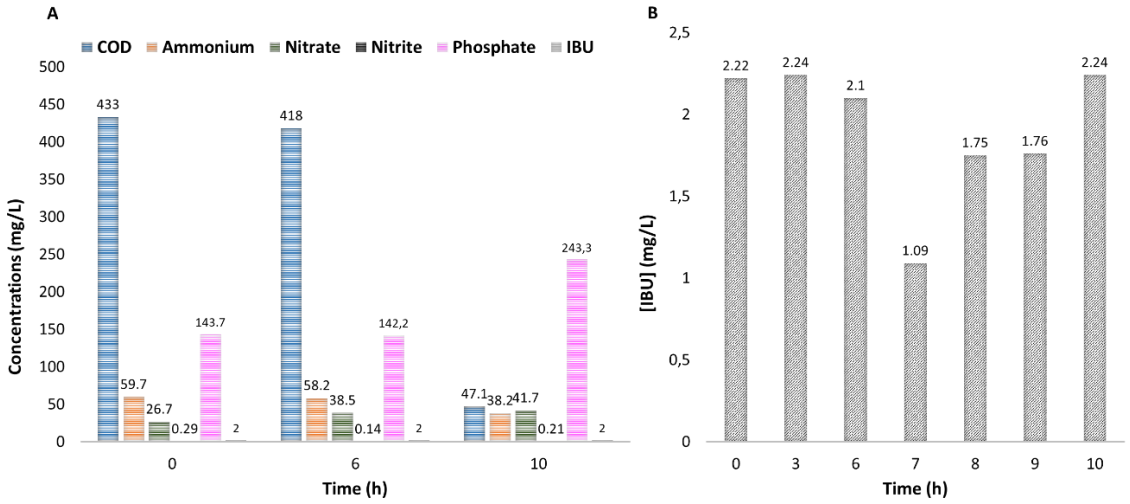


Figure 8.13 IBU (2 mg/L) removal associated with ammonium, nitrate, nitrite, phosphate degradation patterns, after 85 days of SBR operation. Complete cycle of 10 h, 6 h contact time, with 4 h (A) and 2 h (B) aeration. The LOD was of 0.1 mg/L of IBU

As obtained previously, COD, only decreased when aeration started. Also 23% of ammonium was removed, while nitrate and phosphate suffered accumulation. Nitrite was removed in 52%, thus denitrification seemed to occur after 6 hours of anaerobic phase, although after aeration started it was evident that nitrite accumulation occurs. IBU was not degraded by this microbial community in the anaerobic phase, since its concentration remains stable (**Figure 8.13**). On the other hand, in the aerobic phase IBU concentration was halved (from 2.22 to 1.09 mg/L IBU), with its concentration increasing until the end of experiment. A possible explanation to this event, is IBU adsorption to granules with the occurrence of an adsorption/desorption mechanism, that culminates with the release of the drug into the water. This adsorption/desorption mechanism was already reported in IBU and other pharmaceutical studies performed in granular systems (Ferrando-Climent *et al*. 2012; Moreira *et al*. 2015).

The previous system had incorporated an injection pump which together with an aquarium pump introduced water into the system within 2 minutes, and then added some time without aeration to try to recreate an anaerobic feed. In order to see if this last operation mode influence COD removal, the injector pumps were replaced by a peristaltic pump that filled the system in 6 hours at 5 rpm. **Figure 8.14** shows the parameters analysis after adding the peristaltic pump, in a 10-hour cycle, with 6 hours of feeding and 4 hours aeration, after day 128 of SBR operation. Also, IBU was added.

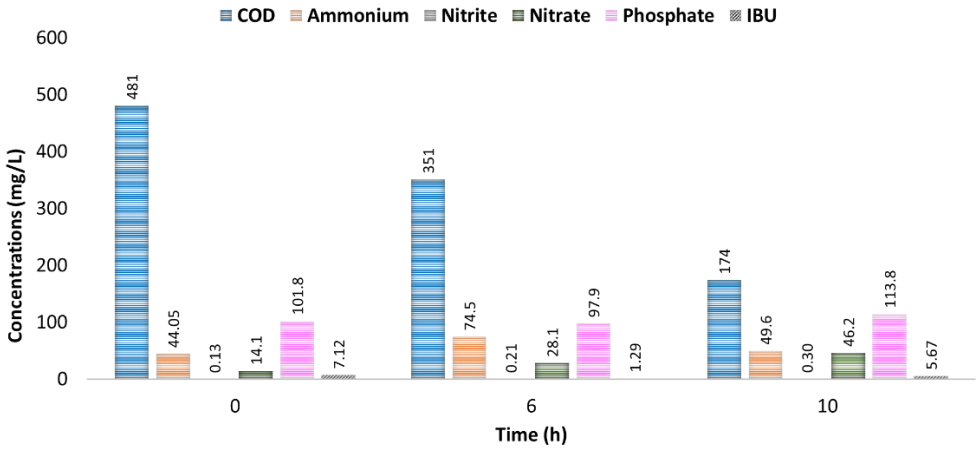


Figure 8.14 COD concentration (mg O_2/L) over the 10-hour cycle, with 6 hours feeding and 4 hours aeration. Use of peristaltic pump to feed the system. IBU (7 mg/L) concentration during the diurnal cycle of 128 days of work. Total cycle time of 10 hours, 6 hours contact time and 4 hours aeration

Figure 8.14 demonstrates that this method was also not effective and suffered from the same effects as already described above. In this case there were no evidence of removal of ammonium, nitrate, nitrite or phosphate. Only COD decreased, however in a lower percentage (64%) in comparison with the other operation system. Ibuprofen removal was 82% after 6 hours and of 20% after 10 hours, therefore with clear evidence of adsorption to sludge that apparently desadsorbed when the aerobic phase started, however it seems that biodegradation may had also occurred (**Figure 8.14**), likely affecting the removal of ammonium or nitrate. The higher percentage of IBU removal, probably by adsorption or biodegradation, may be due to the fact that, the influent with the drug pass through the whole bed of granules slowly allowing higher contact time. The continuous granular system experiments finished at 128 days of operation.

In none of all the experiments performed before, there was COD reduction to the required regulatory limits. Therefore, other assay was performed in order to determine

the required time for anaerobic COD removal. **Figure 8.15** shows COD reduction over a longer period in anaerobic phase. Samples were collected in 3 different cycles of 192 h, 144 h and 196 h.

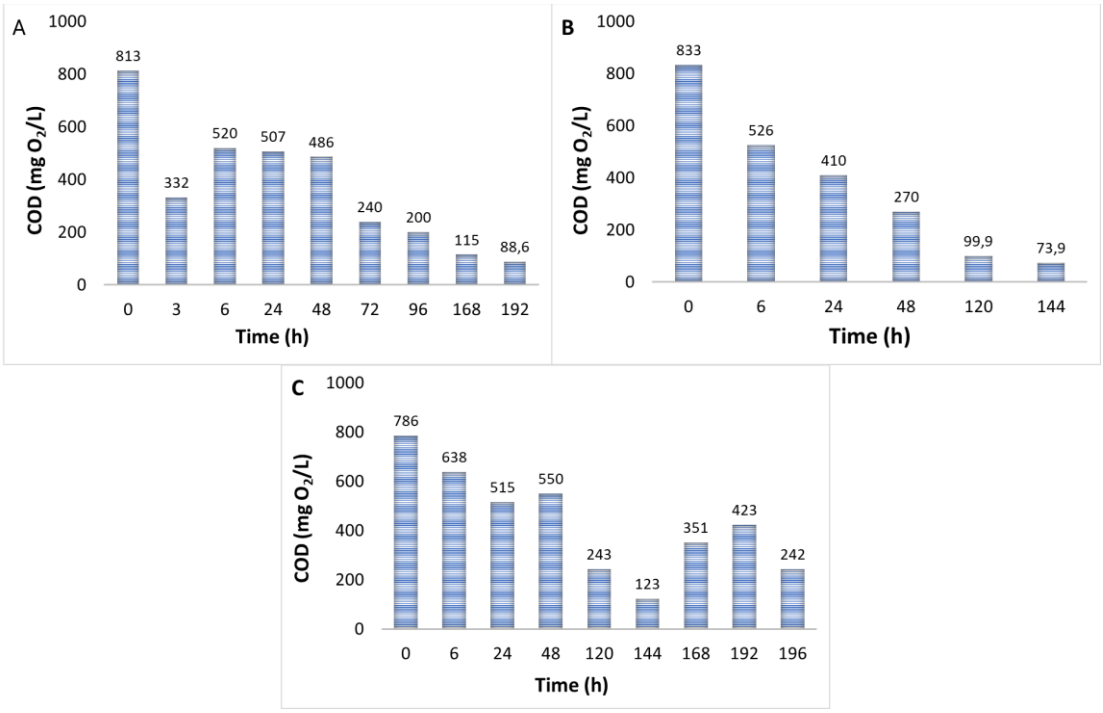


Figure 8.15 COD concentration (mg O_2/L) measured during 3 assays (A, B and C) carried out in the granular sludge adaptation time

The results revealed that in the anaerobic phase of this SBR, COD reduction occurred after several days of contact time ranging from 6 to 8 days.

Figure 8.15A represents the first cycle to be performed, there was a period of adaptation of the microbial community to this new system of 192 hours (8 days), after which COD removal occurred. This conditions somehow favoured the activation of the anaerobic population of the granular sludge. **Figure 8.15B** demonstrated that COD decreased over the days, with an almost complete reduction after 144 h (6 days). **Figure 8.15C** showed an increase in COD after 144 hours (6 days). These values occurred because, as there was no food available to feed the bacterial community, microbial degradation began to occur and subsequent increase in COD values is observed.

Additional studies and system adaptations should be performed in order to optimize this granular SBR.

4. ACHIEVEMENTS OVERVIEW AND FUTURE PERSPECTIVES

In the first two months the reactor was operated at room temperature (23 ± 2ºC) in successive cycles of 168 min (2.8 h, 8.6 cycles daily). Reactor 1 was operated in an aerobic cycle, while reactor 2 was operated in the typical anaerobic/aerobic cycle of aerobic granular sludge reactors. An additional contact time of 42 min was programmed for reactor 2 to prolong contact of influent with the granules due to the short feeding time. After one month of operation, filamentous bulking and slime in the aerobic granules were observed, indicating possible nutrient deficiency. Fungal growth was also observed in the influent containers. Although, acetate (carbon source) was always added in freshly prepared synthetic wastewater, the results showed that acetate of the influent introduced to the SBRs was low, therefore the granules were not receiving the carbon load needed to maintain stability and form. It is possible that degradation in the influent reservoir by opportunists was occurring, as the influent was stored in between cycles.
However, results showed that COD values decreased below regulatory limit of 125 mg O_2/L in 42 min for both aerobic and anaerobic processes, phosphate was slightly removed, and the ammonium concentration started to decrease, while nitrate increased likely due to nitrification (the biological oxidation of ammonium to nitrite followed by the oxidation of the nitrite to nitrate) under aerobic conditions.

To overcome the mentioned problems of acetate degradation, a motorized syringe infusion pump was added to inject the carbon source separately from the micronutrients.

Nevertheless, other problems appeared, maybe due to the aging of granules, such as the non-removal of COD in the anaerobic/anoxic phase. In order to obtain COD removal in this phase, the contact time was increased to a total of 10 hours. Even so, neither COD nor acetate were removed during this period of 6 h contact time. Just only when aeration started (corresponding to 7 h cycle), it was possible to observe the decrease of COD and acetate. After 30 min of the beginning of aeration it was possible to verify the activity of the bacterial community with the decrease of COD below the regulatory limit of 125 mg O_2/L and of acetate below the limits of detection (LOD of 0.05 g/L acetate) of the analytical method used. In this longer cycle there were no evidence of removal of ammonium, nitrate, nitrite or phosphate, maybe due to the addition of 7 mg/L IBU. Although the system was not optimized, it was attempted to test its capacity in the degradation of 7 mg/L IBU in a total cycle of 10 hours. In the anaerobic/anoxic phase Ibuprofen´s adsorption to sludge seems to occur with 82% removal, whereas

degradation by bacteria started after 4 hours of aeration when about 20% of IBU was removed. COD removal was not affected by the addition of IBU. This system is still under optimization. For example, as next step, in order, to promote denitrification, the oxygen could be reduced to 20% by adding a nitrogen source to the reactor, avoiding stratification through the mechanical stirring promoted by the nitrogen aeration. A longer time of acclimatization can be tested using new granules that can be also produced by WTPP sludge. In addition, a metagenomic analysis of the 16S rRNA of the granular population existent in the granular sludge should be performed in order to see which processes are favoured and what can be improved.

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CHAPTER 9

General conclusions and Future perspectives

GENERAL CONCLUSIONS

- **Chloramphenicol and Paracetamol heterogeneous photocatalysis**

• PbS/TiO_2 nanocomposites were successfully synthesized, using biological sulphide produced by SRB in batch cultures during the "Bionanomine" Project. They were used as photocatalysts in the degradation of CAP and paracetamol.

• Drugs degradation was tested by photolysis, in the absence of catalyst and the results indicated that CAP and paracetamol were not completely degraded. Nevertheless, both drugs can be completely degraded in the presence of a catalyst in photoreactor at 450 W and by sunlight exposition (UV and visible light). The presence of the nanocomposite PbS/TiO_2 and TiO_2 increases the reaction rate and the overall conversion. The degradation process follows a pseudo first order degradation.

• The photodegradation of CAP by photolysis was of about 61% and 36%, after 60 min irradiation at 450 W and under sunlight exposition, respectively. In the presence of nanocomposites as catalyst, a high drug removal efficiency of about 96% and 93% was achieved after 60 min and 240 min irradiation, respectively, while using TiO_2 the removal efficiency was about 98% for both photoreactor and sunlight irradiation.

• Paracetamol photocatalysis using PbS/TiO_2 nanocomposites was also successfully performed under sunlight exposition with a high removal of 93%, while paracetamol photodegradation using TiO_2 was complete (about 100%), after 265 min of irradiation. Drug´s removal by photolysis was about 18%.

- **Biodegradation assays**

✓ **Paracetamol**

• The obtained results of paracetamol biodegradation were better in the presence of aerobic (approximately 99.9% of degradation) than in the anaerobic bacterial community (where the highest removal was about 60%).

• The DNA samples corresponding to the best obtained results, where paracetamol degradation was almost complete, were analysed. The results found evidence for a shift in the bacterial community, thus in that cultures were found *Pseudomonas sp*; *Flavobacterium*, *Dokdonella* and *Methylophilus,* which may be involved in paracetamol degradation.

• Assays using bacterial isolates of an aerobic sludge from an oxidation ditch's WWTP suggest for the first time, to our knowledge, that a member of *Bacillus cereus*

group assigned to *Bacillus pacificus* strain MCCC 1A06182 or *Bacillus paranthracis* strain MCCC 1A00395, *[Brevibacterium] frigoritolerans*, *Corynebacterium nuruki* and *Enterococcus faecium* were able to biodegrade paracetamol. A specie from *Bacillus cereus* group degraded below the limits of detection 200 mg/L of paracetamol, apparently without toxic secondary metabolites and removed about 95 ± 5% of 500 mg/L of the drug after 144 h of incubation at 28ºC.

- ✓ **Fluoxetine**
  - **Anaerobic conditions**

• The anaerobic SRB consortium degraded below the limits of detection 20 mg/L and 50 mg/L of FLX as an additional carbon source along with lactate, after 28 and 31 days, respectively. It was also found that this consortium was able to degrade below the LOD, 20 mg/L FLX and removed 70% of 50 mg/L of that drug when used as unique carbon source.

• NFLX was identified as metabolic product during the biodegradation of 50 mg/L FLX, while in the biodegradation of 20 mg/L of FLX, NFLX was not identified but another intermediate metabolite was, the 2,3-dimethyl-5-(trifluoromethyl)benzene-1,4-diol (a terpene with antimicrobial activity) detected, after 28 days of assay.

• The bacteria belonging to the SRB community capable of growing in the presence of FLX as unique carbon source, thus with a possible role in its degradation, were identified as strains from the genera *vadinBC27* wastewater-sludge group, *Macellibacteroidetes*, *Dethiosulfovibrio*, *Bacteroides*, *Tolumonas*, *Sulfuricurvum*, f_*Enterobacteriaceae*_OTU_18 and assumed for the first time as FLX degrading bacteria.

  - **Aerobic conditions**

• In aerobic assays under aeration, in the presence of 20 mg/L FLX, a removal between 83 to 96% of that drug was observed.

• The metabolite NFLX was detected on completion of FLX degradation, but the results suggest its further degradation.

• In the assay of FLX degradation using aerobic sludge and wastewater under aeration, the COD values decreased but remained above the regulatory limit of 125 mg O_2/L.

- The DNA samples corresponding to the best obtained results, where FLX degradation was the best, were analysed. The results found evidence of a shift in the bacterial community; thus, those cultures may be involved in FLX degradation. The aerobic bacterial strains from genera *Aeromonadales*_OTU_14, *Acinetobacter*, *Rheinheimera*, *Shewanella*, *Pseudomonas*, f_*Pseudomonadaceae*_OTU_4914, OTU_24, f_JI49D030_OTU_6171, *Methylobacillus* and *Piscinobacter* were identified and reported for the first time as FLX biodegraders.
- Starting from the sludge previously used for the FLX's biodegradation studies using bacterial communities, six bacterial strains that grew in the presence of FLX were isolated. The bacterial isolates were assigned to *Pseudomonas putida* strain NBRC 14164, *Enterobacter ludwigii* strain EN-119, *Pseudomonas nitritireducens* strain WZBFD3-5A2, *Alcaligenes faecalis* strain NBRC 13111, *Pseudomonas aeruginosa* strain DSM 50071 and *Pseudomonas nitroreducens* strain NBRC 12694 at 100 mg/L of the drug.
- The results showed that *Pseudomonas nitroreducens* displayed the highest removal efficiency of 55 ± 1% at 20 mg/L FLX, after 168 h incubation. As expected, *Pseudomonas* also represents one of the genera with highest final relative abundance in the aerobic bacterial community that degraded FLX.

Although the main mechanism of FLX removal described in the literature is adsorption to sediments or sludge, in the results herein described, biodegradation, either under aerobic or anaerobic conditions, appears to have a major role in the degradation of this drug.

- ✓ **17α-Ethinylestradiol**

- Bacteria with ability to grow in the presence of 50 mg/L of EE2 were isolated from a sludge collected in the oxidation ditch of Faro's Northwest WWTP, being these, *Acinetobacter bouvetii*, *Acinetobacter kookii*, *Pantoea agglomerans* and *Shinella zoogloeoides* species.
- The results showed that *Acinetobacter bouvetii*, *Acinetobacter kookii*, *Pantoea agglomerans* and *Shinella zoogloeoides*, *Pantoea agglomerans* and *Shinella zoogloeoide*s were able to degrade 47 ± 4%, 55 ± 3%, 64 ± 4% and 35 ± 4%, respectively of 13 mg/L EE2 after 168 h at 28 ºC.
- The degradation of EE2 by these isolates resulted in several possible intermediates which are easily biodegraded and less toxic than the parent compound.

The detected compounds were: mannonic acid 1,4-lactone which is a gamma lactone structure, 2,3-dihydroxybenzoic acid, (Z)-aconitic acid, 2-pentenedioic acid an unsaturated acid and 2-butenedioic acid that may have already suffered meta-cleavage of the A ring. Also, 2-butenedioic acid (fumaric acid) which is a dicarboxylic acid and an intermediate metabolite in the TCA cycle was identified.

- **Aerobic granular system**

  The aerobic/anoxic granular sludge SBR system implemented in this work is still under optimization.

  Although the system was not optimized, its ability to biodegradade IBU has been tested in the presence of 7 mg/L IBU in a total cycle of 10 hours. From these tested conditions it can be inferred that IBU may suffer adsorption to sludge, in the anaerobic/anoxic phase, with 82% removal, whereas degradation by bacteria started after 4 hours of aeration when about 20% of IBU was removed. COD removal was not affected by the addition of IBU.

FUTURE PERSPECTIVES

The present study was performed aiming a better knowledge about the ability and behaviour of environmental and naturally occurring in WWTP's bacterial communities and isolates for the biodegradation of some emerging relevant pharmaceuticals under study. From the obtained results with the performed studies, it is expected that they can help into found new, efficient, and cost-effective strategies of wastewater treatment.

Thus, based on the obtained results of this thesis, the following recommendations are suggested for future study to onward increase the current knowledge on this subject:

a) This study tested higher concentrations of pharmaceuticals than those that have been measured in wastewaters and surface waters, in order to increase experimental precision and analytical accuracy. However, experiments using lower concentrations of pharmaceutical compounds in the range of ng/L to µg/L should be carried out to study the behaviour of bacteria. Nevertheless, according to the BioWin predictive model it is expected/can be inferred that these bacteria should be faster and more effective in drug's biodegradation;

b) Biodegradation studies of these and other drugs through the addition of bacterial isolates that have demonstrated degradation capacity, to already existing bacterial communities (bio-augmentation) (see how such inclusion enhances the removal of the drug);
c) Biostimulation/bio-augmentation experiments that can be performed by adding bacterial biomass, produced in the laboratory, in a pilot system mimicking an already implemented system (*e.g.* oxidation ditch), in order to improve wastewater treatment processes;
d) Production of a "super-sludge", or "super-granule" by adding for example phosphate-accumulating bacteria (PAOs) to the existing population and would, in the latter case, make a more efficient anoxic/aerobic system capable of reducing COD, nitrates, nitrites, phosphorus and pollutants (drugs) in one step;
e) Implementation of a pilot scale biological reactor in a WWTP using as inoculum the studied bacteria with biodegradation abilities or the “super-sludge”/“super-granules” that would be produced, with a controlled contact time, in order to improve the wastewater treatment and allowing an excellent efficiency in the degradation of COD, pharmaceuticals and eventually other toxic organic compounds, nitrates, nitrites, phosphorous;
f) In addition, it would be interesting to carry out enzymatic and genetic studies that would allow to deepen and clarify the metabolic processes that occur during the degradation of these drugs by the obtained bacteria.

ANNEXES

Annex 1 (CHAPTER 3)

Table S1 Percentages of 16S rRNA amplicons per OTU for the 25 most abundant bacteria, taxonomic classifications assigned using the RDP classifier as described in Materials and methods (CHAPTER 4)

Percentages of 16S rRNA amplicons per OTU for the 25 most abundant bacteria					Taxonomic classifications assigned using the RDP classifier as described in materials and methods.						OTU (see GenBank accession numbers in Online Resource 2)
Inoculum - sludge from WWTP oxidation ditch	Culture in MSM - Day 1	Culture in MSM - Day 2	Culture in MSM - Day 3	Culture in MSM - Day 6	Kingdom	Phylum	Class	Order	Family	Genus	
0.00	0.00	0.02	0.08	0.05	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	OTU_144
0.01	0.00	0.00	0.46	0.21	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	OTU_1497
0.03	0.02	0.01	0.14	0.27	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	OTU_16
0.00	0.00	0.00	0.00	0.01	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	OTU_166
0.00	0.00	0.00	0.00	0.00	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	OTU_1694
0.01	0.01	0.01	0.01	0.00	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	OTU_1739

0.01	0.01	0.00	0.01	0.02	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	OTU_67
0.04	**0.00**	**0.04**	**7.17**	**20.63**	**Bacteria**	**Proteobacteria**	**Gammaproteobacteria**	**Pseudomonadales**	**Pseudomonadaceae**	**Pseudomonas**	**OTU_7**
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1030
0.01	0.00	0.01	0.01	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1043
0.00	0.01	0.00	0.00	0.02	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1081
0.00	0.00	0.00	0.01	0.58	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_112
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1162
0.00	0.00	0.00	0.00	0.03	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1203
0.23	0.25	0.19	0.22	0.15	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_130
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1322
0.00	0.00	0.00	0.00	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1376
0.01	0.01	0.01	0.01	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1378
0.00	0.00	0.00	0.00	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1419
0.01	0.02	0.02	0.02	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1506
0.00	0.00	0.00	0.00	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1507
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1540
0.00	0.00	0.00	0.01	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1636
0.00	0.00	0.00	0.01	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1672
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1687
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1755
0.01	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1788
0.00	0.00	0.00	0.01	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1845
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1924
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_1992
0.17	**0.63**	**0.93**	**1.67**	**4.89**	**Bacteria**	**Bacteroidetes**	**Flavobacteriia**	**Flavobacteriales**	**Flavobacteriaceae**	**Flavobacterium**	**OTU_20**
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2141
0.00	0.00	0.01	0.01	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2179
0.00	0.00	0.00	0.01	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2185
0.26	0.41	0.35	0.38	0.25	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_220
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2383

0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2388
0.00	0.00	0.00	0.00	0.02	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2538
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2595
0.00	0.00	0.00	0.00	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2655
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2711
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_2807
0.11	0.13	0.12	0.09	0.06	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_281
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_436
0.09	0.02	0.01	0.02	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_451
0.02	0.05	0.04	0.10	0.12	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_453
0.03	0.03	0.05	0.03	0.02	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_525
0.02	0.02	0.02	0.01	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_562
0.61	0.82	0.73	0.94	0.62	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_60
0.01	0.02	0.02	0.02	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_602
0.02	0.02	0.02	0.03	0.02	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_776
0.00	0.02	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_803
0.01	0.01	0.02	0.02	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_804
0.00	0.00	0.00	0.00	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_861
0.03	0.01	0.01	0.01	0.01	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	OTU_976
2.86	5.28	4.68	3.55	2.68	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae		OTU_6
1.28	2.10	2.63	1.80	1.18	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	PHOS-HE31	OTU_15
0.46	0.74	0.94	1.04	0.86	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	PHOS-HE31	OTU_239
0.43	0.48	0.53	0.53	0.31	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	PHOS-HE31	OTU_82
2.25	2.63	3.62	3.91	2.07	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	PHOS-HE51		OTU_9
1.41	**2.11**	**3.01**	**3.16**	**3.67**	**Bacteria**	**Proteobacteria**	**Gammaproteobacteria**	**Xanthomonadales**	**Xanthomonadaceae**	**Dokdonella**	**OTU_10**
0.00	0.01	0.01	0.01	0.01	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dokdonella	OTU_1065
0.00	0.00	0.01	0.01	0.02	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dokdonella	OTU_1410
0.07	0.08	0.10	0.10	0.10	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dokdonella	OTU_245
0.00	0.00	0.00	0.00	0.00	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dokdonella	OTU_2759
4.97	1.70	1.25	0.58	0.57	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	QEDR3BF09	OTU_11

0.01	0.01	0.01	0.06	0.02	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	QEDR3BF09	OTU_1436
0.00	0.00	0.01	0.04	0.02	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	QEDR3BF09	OTU_1580
0.41	0.17	0.13	0.07	0.05	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	QEDR3BF09	OTU_173
0.53	0.23	0.24	0.06	0.05	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	QEDR3BF09	OTU_182
0.09	0.09	0.10	0.03	0.03	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	QEDR3BF09	OTU_288
0.02	0.09	0.06	0.06	0.06	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	QEDR3BF09	OTU_318
0.03	0.05	0.03	0.12	0.09	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	QEDR3BF09	OTU_336
0.29	0.26	0.24	0.19	0.16	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_105
0.28	0.24	0.22	0.04	0.13	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_114
0.01	0.00	0.01	0.00	0.00	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_1165
0.27	0.25	0.21	0.08	0.04	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_133
0.30	0.21	0.22	0.13	0.08	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_158
0.23	0.11	0.13	0.06	0.04	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_196
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_1966
0.69	0.64	0.74	0.23	0.29	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_41
0.68	0.58	0.48	0.14	0.23	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_72
0.02	0.01	0.00	0.01	0.00	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_828
0.59	0.51	0.44	0.26	0.05	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	CYCU-0281	OTU_90
0.00	0.02	0.02	0.10	0.02	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Azospira	OTU_26
6.03	1.25	1.27	0.71	1.50	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Azospira	OTU_29
0.03	0.00	0.00	0.01	0.00	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Uliginosibacterium	OTU_1154
0.00	0.00	0.00	0.01	0.01	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Uliginosibacterium	OTU_1337
2.63	3.43	2.13	1.94	0.22	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Uliginosibacterium	OTU_17
2.06	2.17	1.63	0.71	0.45	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	188up	OTU_32
0.75	1.13	0.92	0.29	0.17	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	188up	OTU_944
0.01	0.00	0.01	0.02	0.06	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	OTU_123
0.01	0.01	0.00	0.00	0.01	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	OTU_1379
0.00	0.00	0.00	0.00	0.00	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	OTU_2476
0.00	0.00	0.00	0.00	0.01	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	OTU_2666
0.40	0.09	0.11	0.05	0.04	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	OTU_308

0.01	0.01	0.01	0.01	0.07	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	OTU_33
0.02	0.01	0.02	0.01	0.01	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	OTU_551
0.34	0.27	0.35	0.30	0.18	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9 Marine group	PHOS-HE28	OTU_117
0.00	0.00	0.00	0.01	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9 Marine group	PHOS-HE28	OTU_1487
0.27	0.44	0.57	0.36	0.45	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9 Marine group	PHOS-HE28	OTU_215
0.13	0.23	0.22	0.16	0.15	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9 Marine group	PHOS-HE28	OTU_232
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9 Marine group	PHOS-HE28	OTU_2349
0.11	0.13	0.13	0.11	0.09	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9 Marine group	PHOS-HE28	OTU_241
0.16	0.13	0.21	0.14	0.11	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9 Marine group	PHOS-HE28	OTU_478
0.35	0.34	0.54	0.38	0.43	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9 Marine group	PHOS-HE28	OTU_73
0.27	0.34	0.37	0.18	0.20	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	NS9 Marine group	PHOS-HE28	OTU_94
2.42	2.20	2.14	0.64	1.16	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	MK04	OTU_13
0.00	0.00	0.00	0.00	0.00	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	MK04	OTU_1889
0.17	0.20	0.09	0.22	0.37	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas	OTU_202
0.04	0.06	0.07	0.03	0.04	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas	OTU_357
0.59	0.76	0.59	0.44	0.80	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas	OTU_36
0.01	0.01	0.02	0.01	0.04	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas	OTU_749
0.11	0.32	0.20	1.45	0.70	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas	OTU_80
0.00	**0.08**	**0.34**	**0.43**	**3.83**	**Bacteria**	**Proteobacteria**	**Betaproteobacteria**	**Methylophilales**	**Methylophilaceae**	**Methylophilus**	**OTU_23**
0.00	0.00	0.00	0.00	0.00	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	OTU_1102
0.00	0.00	0.00	0.00	0.02	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	OTU_207
0.00	0.00	0.00	0.00	0.03	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	OTU_35
0.02	0.30	0.32	0.21	0.25	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_107
0.10	0.27	0.24	0.05	0.08	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_129
0.05	0.08	0.06	0.05	0.02	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_256
0.03	0.08	0.09	0.04	0.04	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_305
0.02	0.08	0.07	0.01	0.03	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_313
0.14	1.20	1.18	0.57	1.05	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_37
0.02	0.03	0.03	0.02	0.08	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_418
0.02	0.05	0.06	0.02	0.05	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_532

0.00	0.00	0.01	0.01	0.02	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_819
0.00	0.01	0.01	0.01	0.00	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	OTU_901
0.61	1.13	1.00	1.12	1.69	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax	OTU_332
0.14	0.05	0.06	0.06	0.05	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax	OTU_744
0.00	0.00	0.00	0.00	0.00	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	OTU_1356
0.00	0.00	0.01	0.00	0.00	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	OTU_2145
0.00	0.00	0.00	0.00	0.00	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	OTU_2324
0.01	0.00	0.00	0.01	0.00	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	OTU_2723
0.21	0.06	0.07	0.08	0.03	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	OTU_341
0.04	0.01	0.01	0.07	0.01	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	OTU_482
1.93	0.73	0.72	1.14	0.19	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	OTU_51
0.09	0.02	0.01	0.30	0.01	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	OTU_520
0.05	0.03	0.01	0.02	0.01	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter	OTU_569
0.01	0.01	0.02	0.01	0.00	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Lautropia	OTU_1341
0.08	0.15	0.24	0.10	0.48	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Lautropia	OTU_189
0.41	0.95	1.13	0.73	1.01	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Lautropia	OTU_42
0.04	0.05	0.09	0.04	0.04	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Lautropia	OTU_567
0.02	0.02	0.03	0.02	0.00	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Lautropia	OTU_687
0.01	0.01	0.02	0.01	0.02	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Lautropia	OTU_917
0.00	0.00	0.00	0.02	0.00	Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	SBR1029	OTU_1511
0.00	0.00	0.00	0.00	0.00	Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	SBR1029	OTU_2778
0.23	1.89	1.57	0.58	0.20	Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	SBR1029	OTU_30
0.03	0.01	0.04	0.10	0.01	Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae	SBR1029	OTU_433
0.28	0.79	0.97	1.18	1.73	Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	uncultured	OTU_28
0.00	0.00	0.00	0.00	0.00	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiales Incertae Sedis	Candidatus Odyssella	OTU_1491
0.00	0.00	0.00	0.00	0.01	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiales Incertae Sedis	Candidatus Odyssella	OTU_1762
0.15	0.85	1.15	0.67	1.93	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiales Incertae Sedis	Candidatus Odyssella	OTU_21
0.00	0.00	0.00	0.00	0.00	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiales Incertae Sedis	Candidatus	OTU_2473

											Odyssella	
0.00	0.00	0.01	0.01	0.00	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_1283
0.03	0.00	0.01	0.01	0.01	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_1364
0.01	0.00	0.00	0.00	0.00	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_1890
0.00	0.00	0.00	0.00	0.00	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_2002
0.00	0.01	0.00	0.00	0.00	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_2052
0.17	0.14	0.08	0.22	0.05	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_283
0.07	0.11	0.12	0.14	0.03	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_297
0.03	0.07	0.08	0.12	0.03	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_376
0.01	0.01	0.02	0.03	0.01	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_676
0.37	0.71	0.74	0.96	0.34	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	OTU_84

Table S2 List of OTUs and respective GenBank Numbers (MG554745 to MG557554) (CHAPTER 4)

List of OTUs and respective GenBank Numbers (MG554745 to MG557554):

OTU_1 (MG554745); OTU_2 (MG554746); OTU_3 (MG554747); OTU_4 (MG554748); OTU_5 (MG554749); OTU_6 (MG554750); OTU_7 (MG554751); OTU_8 (MG554752); OTU_9 (MG554753); OTU_10 (MG554754); OTU_11 (MG554755); OTU_12 (MG554756); OTU_13 (MG554757); OTU_14 (MG554758); OTU_15 (MG554759); OTU_16 (MG554760); OTU_17 (MG554761); OTU_18 (MG554762); OTU_19 (MG554763); OTU_20 (MG554764); OTU_21 (MG554765); OTU_22 (MG554766); OTU_23 (MG554767); OTU_24 (MG554768); OTU_25 (MG554769); OTU_26 (MG554770); OTU_27 (MG554771); OTU_28 (MG554772); OTU_29 (MG554773); OTU_30 (MG554774); OTU_31 (MG554775); OTU_32 (MG554776); OTU_33 (MG554777); OTU_34 (MG554778); OTU_35 (MG554779); OTU_36 (MG554780); OTU_37 (MG554781); OTU_38 (MG554782); OTU_39 (MG554783); OTU_40 (MG554784); OTU_41 (MG554785); OTU_42 (MG554786); OTU_43 (MG554787); OTU_44 (MG554788); OTU_45 (MG554789); OTU_46 (MG554790); OTU_47 (MG554791); OTU_48 (MG554792); OTU_49 (MG554793); OTU_50 (MG554794); OTU_51 (MG554795); OTU_52 (MG554796); OTU_53 (MG554797); OTU_54 (MG554798); OTU_55 (MG554799); OTU_56 (MG554800); OTU_57 (MG554801); OTU_58 (MG554802); OTU_59 (MG554803); OTU_60 (MG554804); OTU_61 (MG554805); OTU_62 (MG554806); OTU_63 (MG554807); OTU_64 (MG554808); OTU_65 (MG554809); OTU_66 (MG554810); OTU_67 (MG554811); OTU_68 (MG554812); OTU_69 (MG554813); OTU_70 (MG554814); OTU_71 (MG554815); OTU_72 (MG554816); OTU_73 (MG554817); OTU_74 (MG554818); OTU_75 (MG554819); OTU_76 (MG554820); OTU_77 (MG554821); OTU_78 (MG554822); OTU_79 (MG554823); OTU_80 (MG554824); OTU_81 (MG554825); OTU_82 (MG554826); OTU_83 (MG554827); OTU_84 (MG554828); OTU_85 (MG554829); OTU_86 (MG554830); OTU_87 (MG554831); OTU_88 (MG554832); OTU_89 (MG554833); OTU_90 (MG554834); OTU_91 (MG554835); OTU_92 (MG554836); OTU_93 (MG554837); OTU_94 (MG554838); OTU_95 (MG554839); OTU_96 (MG554840); OTU_97 (MG554841); OTU_98 (MG554842); OTU_99 (MG554843); OTU_100 (MG554844); OTU_101 (MG554845); OTU_102 (MG554846); OTU_103 (MG554847); OTU_104 (MG554848); OTU_105 (MG554849); OTU_106 (MG554850); OTU_107 (MG554851); OTU_108 (MG554852); OTU_109 (MG554853); OTU_110 (MG554854); OTU_111 (MG554855); OTU_112 (MG554856); OTU_113 (MG554857); OTU_114 (MG554858); OTU_115 (MG554859); OTU_116 (MG554860); OTU_117 (MG554861); OTU_118 (MG554862); OTU_119 (MG554863); OTU_120 (MG554864); OTU_121 (MG554865); OTU_122 (MG554866); OTU_123 (MG554867); OTU_124 (MG554868); OTU_125 (MG554869); OTU_126 (MG554870); OTU_127 (MG554871); OTU_128 (MG554872); OTU_129 (MG554873); OTU_130 (MG554874); OTU_131 (MG554875); OTU_132 (MG554876); OTU_133 (MG554877); OTU_134 (MG554878); OTU_135 (MG554879); OTU_136 (MG554880); OTU_137 (MG554881); OTU_138 (MG554882); OTU_139 (MG554883); OTU_140 (MG554884); OTU_141 (MG554885); OTU_142 (MG554886); OTU_143 (MG554887); OTU_144 (MG554888); OTU_145 (MG554889); OTU_146 (MG554890); OTU_147 (MG554891); OTU_148 (MG554892); OTU_149 (MG554893); OTU_150 (MG554894); OTU_151 (MG554895); OTU_152 (MG554896); OTU_153 (MG554897); OTU_154 (MG554898); OTU_155 (MG554899); OTU_156 (MG554900); OTU_157 (MG554901); OTU_158 (MG554902); OTU_159 (MG554903); OTU_160 (MG554904); OTU_161 (MG554905); OTU_162 (MG554906); OTU_163 (MG554907); OTU_164 (MG554908); OTU_165 (MG554909); OTU_166 (MG554910); OTU_167 (MG554911); OTU_168 (MG554912); OTU_169 (MG554913); OTU_170 (MG554914); OTU_171 (MG554915); OTU_172 (MG554916); OTU_173 (MG554917); OTU_174 (MG554918); OTU_175 (MG554919); OTU_176 (MG554920); OTU_177 (MG554921); OTU_178 (MG554922); OTU_179 (MG554923); OTU_180 (MG554924); OTU_181 (MG554925); OTU_182 (MG554926); OTU_183 (MG554927); OTU_184 (MG554928); OTU_185 (MG554929); OTU_186 (MG554930); OTU_187 (MG554931); OTU_188 (MG554932); OTU_189 (MG554933); OTU_190 (MG554934); OTU_191 (MG554935); OTU_192 (MG554936); OTU_193 (MG554937); OTU_194 (MG554938); OTU_195 (MG554939); OTU_196

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Annex 2 (CHAPTER 4)

Table S3 Sequence contigs (treated data) obtained with the BioEdit software that resulted from DNA Sanger sequencing of each bacterial isolate

Isolate	BioEdit Contig Sequences
1	>Contig-0 AGAGTTTGATCCTGGCTCAGAAGTCGTAACAAGGTAACCAGTTAGAGTTTGATCCTGGCTCAGAAGTCGTAACAGGGTAACCTGCCCATATTACTGGGATAACTCCGGGAAACCGGG GCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAG GCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGAC GGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACG GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAA CCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTT TCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAG TGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAA CGCGAAGAACCTTAGCCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGT TGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATC ATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAAC TCGCCTACATGAAGCTGGAATCGGTAGTAATCGCGGATCAGCAAGTCGTAGCAAGGTAACCTGTTAGAGTTTGATCCTGGCTCAGAAGTCGTAACAAGGTAACCAGTTAGAGTTTGA TCCTGGCTCAGAAGTCGTAACAAGGTAACCCAGGTT
2	>Contig-0 ACTGCAGTCGAGCGGAGTTATTTTGAAAGCTTGCTTTCAAAATAACTTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCCTTCAGACTGGGATAACTACCGGAAACGGTA GCTAATACCGGATAATTTCTTTTTTCTCCTGAGAGAAGAATGAAAGACGGAGCAATCTGTCACTGAGGGATGGGCCTGCGGCGCATTAGCTAGTTGGTGGGGTAACGGCCCACCAAG GCGACGATGCGTAGCCGACCTGAGAGGGTGAACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGAC GGAGCAACGCCGCGTGAGTGATGAAAGGTTTTCGGATCGTAAAGCTCTGTTGCCAGGGAAGAACGTCCCGGTAGAGTAACTGCTACCGGGAGTGACGGTACCTGAGAAGAAAGCCC CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCTATTTAAGTCTGGTGTTTAAACCTTGGGCTC AACCTGAGGTCGCACTGGAAACTGGGTGGCTTGAGTACAGAAGAGGAAAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACT TTCTGGGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGGTTTCGATACCCTT GGTGCCGAAGTTAACACAGTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTAATTCGAAGC AACGCGAAGAACCTTACCACGTCTTGACATCCCTCTGAATCCGCTAGAGATAGCAGCGGCCTTCGGGACAGAGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATG TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAACTTTAGTTGCCAGCAGGTAATGCTGGGCACTCTAGAGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAAT CATCATGCCCCTTATGACCTGGGCTACACACGTACTACAATGGCCGGTACAACGGGAAGCGAAGCCGCGAGGTGGAGCCAATCCCATCAAAGCCGGTCTCAGTTCGGATTGCAGGC TGCAACTCGCCTGCATGAAGTCGGAATTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTACAACACCC GAAGTCGGTGGGGTAACCCGCAAGGGAGCCAG
3	>Contig-0 TACTGCAAGTCGAGCGAATCGATGGGAGCTTGCTCCCTGAGATTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTA ATACCGGATACGTTCTTTTCTCGCATGAGAGAAGATGGAAAGACGGTTTACGCTGTCACTTATAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGA CGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAG CAACGCCGCGTGAACGAAGAAGGCCTTCGGGTCGTAAAGTTCTGTTGTTAGGGAAGAACAAGTACCAGAGTAACTGCTGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAA CTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTCCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTG

	GAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCAAGTGTAGCGGTGAAATGCGTAGAGATTTGGAGGAACACCAGTGGCGAAGGCGACTTTCTGG TCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTG CAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG AAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGG GTTAAGTCCCGCAACGAGCGCAACCCTTGAATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCA TGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCTGCAAACCTGCGAAGGTAAGCGAATCCCATAAAGCCATTCTCAGTTCGGATTGCAGGCTGCAACT CGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCG GTGAGGTAACCTTCATGGAGCCAGCCG
4	>Contig-0 AGAGTTTGATCCTGGCTCAGAAGTCGTAACAAGGTAACCAGTATAGAGTTTGATCCTGGCTCAGAAGTCGTAACAAGGTGACCAGCTATGGTTTTCTGGAGAAGCCTGGGAAACTGG GTCTAATACCGGATAGGACCATTGGTTGGTACTGGTGGTGGAAAGTTTTTCGGTGCAGGATGAGCTCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGGCGACGG GTAGCCGGCCTGAGAGGGTGGACGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGC CGCGTGGGGGATGACGGCCTTCGGGTTGTAAACTCCTTTCAACCATGACGAAGCTTTTGTGACGGTAGTGGTAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATAC GTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTCGTCTGTGAAATTCCGGGGCTCAACTCCGGGCGTGCAGGCGATACGGGCATAAAC TTGAGTGCTGTAGGGGAGACTGGAATTCCTGGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGGTCTCTGGGCAGTAACTGACGCTGAGGAGC GAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGGTGGGCGCTAGGTGTGGGGGTCTTCCACGACTTCTGTGCCGTAGCTAACGCATTAAGCGCCCC GCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGCTTGAC ATGTACCGGATCGGCGCAGAGATGTGTCTTCCCTTGTGGTCGGTATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC TTGTCCTGTGTTGCCAGCACGTTGTGGTGGGGACTCACGGGAGACCGCCGGGGTTAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCCAGGGCTTCACAC ATGCTACAATGGTCGGTACAGAGGGTTGCTACACCGTGAGGTGGTGCTAATCTCTTAAAGCCGGTCTCAGTTCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAGTTGGTAG TAATCGCAGATCAGCAAGGCGGAGCAGAGTAACCTGTTAGAGTTTGATCCCGGCTCAGAAGTCGTAACAAGGTAACCTGTTAGAGTTTGATCCTGGCTCAGAAGTCGTAACAA
5	>Contig-0 CTGCAAGTCGTACGCTTTTTCTTTCACCGGAGCTTGCTCCACCGAAAGAAAAGGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAA ACAGGTGCTAATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTC ACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAG TCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAA GCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCG GCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGG CGACTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCC CTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG AAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAAGTGACAGGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGT GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTTAGTTGGGCACTCTAGCAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA AATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTCGCGAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTGTAGG CTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAAAGTTTGTAACATC CCGAAGTCGGTGAGGTAACCTTTTTGGAGCCAGCCGC
6	>Contig-0 TGATCCTGGCTGACAAGTCGTAGCAAGGTAACCACGGGTGAAGTAACGCGTGGGAATCTACCATTTGCTACGGAACAACTCCGGGAAACTGGAGCTAATACCGTATGTGCCCTAAC GGGGAAAGATTTATCGGCAAATGATGAGCCCGCGTTGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACACTGGG ACTGAGACACGGCCCAGACTCCTACGGGAAGGCAGCAGTGGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAG CTCTTTCACCCGGTGAAGATAATGACGGTAACCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAA AGCGCACGTAGGCGGACTTTTAAGTCAGGGGTGAAATCCCGGGGCTCAACCCCGGAACTGCCTTTGATACTGGAAGTCTTGAGTATGGTAGAGGTGAGTGGAATTCCGAGTGTAGA GGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACCATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC CACGCCGTAAACGATGAATGTTAGCCGTCGGGGAGTTTACTCTTCGGTGGCGCAGCTAACGCATTAAACATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTG

	ACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCCCTTGACATACCGGTCGCGGACACAGAGATGTGTCTTTCAGTTCGGCTGG ACCGGATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGG GGACTGCCGGTGAATAAGCCGAGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGTGGTGACAGTGGGCAGCGAGCACGC GAGTGTGAGCTAATCTCCAAAAGCCATCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCG GGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTTTATCCGAAGGCGCTGTGCTAACCGCAAGGAGGCAGG
7	>Contig-0 AGCTGTGCTCCGACGGTTACCTCACCGGAACTTCGTGGTAGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCGTGCTGATCCGCGA TTACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAGAAGCTTTAAGAGATTAGCTTAGCCTCGCGACTTCGCAACTCGTTGTACTTCCCATTGTAGC ACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTTGCTAGAGTGCCCAACTGAATGATGGCAACTAACAATAA GGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTTCACGACACGAGCTGACGACAACCCATGCACCACCTGTCACTTTGCCCCCGAAGGGGAAGCTTCTATCTCTAGAGTGATCAA AGGATGTCAAGACCTGGTAAGGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCA GGCGGAGTGCTTAATGCGTTAGCTGCAGCACTGAAGGGCGGAAACCCTCCAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTT TCGAGCCTCAGCGTCAGTTACAGACCAGAGAGCCGCCTTCGCCACTGGTGTTCCTCCATATATCTACGCATTTCACCGCTACACATGGAATTCCACTCTCCTCTTCTGCACTCAAGTCT CCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCCACCTACGTATTAC CGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGGTTAGATACCGTCAAGGGATGAACAGTTACTCTCATCCTTGTTCTTCTCTAACAACAGAGTTTTACGATCCGAAAACCTTC TTCACTCACGCGGCGTTGCTCGGTCAGACTTTCGTCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAATGTGGCCGATCACCCTCTCAG GTCGGCTATGCATCGTGGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCACCGCGGGTCCATCCATCAGCGACACCCGAAAGCGCCTTTCAAATCAAAACCATGCGGTTTTG ATTGTTATACGGTATTAGCACCTGTTTCCAAGTGTTATCCCCTTCTGATGGGCAGGTTACCCACGTGTTACTCACCCGTTCGCCACTCCTCTTTTTAACCTGGAGCTGGTTACCTTGGA ACGACTTCTGAGCCAGGATCAAACT
8	>Contig-0 CGCGCGTGCGCGTCGCACGCTTTTTCTTTCACCGCCGCTTGCTCCACCCAAAGAAAAAGAGTGGCGAACGGTTGATTAACACGTGCAAAAACCCGCTCATCAGAAGGGGATGCACTT TTCTTAGGTGCCGGTATTGTCTCCCGAGTAAAACCGCATGCCTTTACTTTGAAAGGCGCTTTTGCGGCACTGATGAATGGACCCGCGCTGCATTTCCTAGGTGGTGGGGTACCCGGGC ACCAAGGCAAGCGTGCATCTTCGACCTGAGAGGCTGACCGCCCACATTGGTACTGAGCCAGATCAAACTCTTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCT GACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGGAAGGAACCAAGGGATGAGGAGTAAAATGTTCATCCCTTGGACGGTATTCTAACCA GAAAAGGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGACGTAAAAGCGAGCGCAGGCGGTTTCTAAAGTCTGATGTGAA AGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAAACTTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAG TGGCGAAGGCGGCTCTCTTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGA GGGTTTCCGCCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCTCGCCTGGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGGGCCCGCACAAGCGGTGGAG CATGGGGGTTTTAATTCAAACCAACCCAAAAAACCTTACCAGGGTCTTGACATCCCTTTGGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAGGTGGTGCATGGT TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTTAGTTGGGCACTCTAGCGAGACTGCCGGTGACAAACCGGAG GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTCGCGAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTT CTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGAGCCTTGATCCTGGCTCAGAAGTCA CCACAAGGTAACCAGTCAGAGTTTGATCCTGGCTCAGAAGTCGTAACAAGGTAACTCAG
9	>Contig-0 TTGCATGTCGAACGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAAGAGGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAA ACAGGTGCTAATACCGTATAACAATCAAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTC ACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAG TCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCCAGAAA GCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCG GCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGG CGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCC CTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG AAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGT GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTCAGTTGGGCACTCTAGCAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTC AAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTTGCGAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTGCAG

	GCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACTC CCGAAGTCGGTGAGGTAACCTTTGGAGCACAG
10	>Contig-0 AGTTTGATCCTGGCTCAGAAGTCGTAACAAGGTAACCAGCTTCAAGAGTTTGATCCAGGATCGGCAAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACT TGGAAACAGGTGCTAATACCGTATAACAATCAAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAAC GGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGAC GAAAGTCTGACCGAGAAACCCCGGCGTGAGTGAAGAAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGTTCATTCCCTTGACGGTATTCTA ACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTG AAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCA GTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGA GGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGT GGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACCTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGAACAGGTGGTGCATGGTTGTCGTCA GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTCAGTTGGGCACTCTAGCAAGACTGCCGGTGACAAACCGGAGGAAGGTGG GGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTTGCGAAGTCGCGAGGTTAAGCTAATCTCTTAAAGCTTCTCTCAGTT CGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCTGGCCTTGTACACACCGCCCGTCACACCACAAGGT AATCAGTTAGAGTTTGATCCTGGCTCAGAAGTCGTAACAAGGTAACT
11	>Contig-0 TTTGGATCCTCGGCTGCTTTGTTCTTTCACCGGGAGCTAGCTCCACCGAAAGAAAAGGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTG GAAACAGGTGCTAATACCGTATAACAATAGAAACCGCATGGTTTCTATTTGAAAGGCGCTTTTGCGTCACTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCT CACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAA GTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGGATGAGAGTAAAATGTTCATCCCTTGACGGTATCTAACCAGA AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCC CCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGA AGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTC CGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAA TTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTTAGTTGGGCACTCTAGCGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGT CAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTCGCGAAGTCGCGAGGCTAAGCTAATCTCTTAAAGCTTCTCTCAGTTCGGATTGTA GGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACA CCCGAAGTCGGTGAGGTAACCTTTGGAGCACAGCCGCAG
12	>Contig-0 ATGTTGCAACCTCGAGCGAATGGTATTAAGCAGCTTGCTCATTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACC GGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACC AAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCT GACGGAGCAACGCCGCGTGAGTGATGAAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGC CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGG CTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGC GACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCC CTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG AAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGA GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAA ATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGC TGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACATCA CGCGAAGTCGCGTGGGGTAACCTTTTTGGAGCCAGCCG

Table S4 Bacterial identification using 16S rRNA gene sequences of the twelve bacterial isolates from an aerobic sludge with ability to grow in the presence of paracetamol as unique carbon source

Isolate	Domain (rdp classifier)	Phylum (rdp classifier)	Class (rdp classifier)	Order (rdp classifier)	Family (rdp classifier)	Genus (rdp classifier)	Confidence threshold (rdp classifier)	Query Length (NCBI BLAST)	Query (NCBI BLAST)	% Identity (NCBI BLAST)	Specie (NCBI BLAST)	Accession (NCBI BLAST)
1	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	80%	1434	87%	99.68%	*Bacillus pacificus* strain MCCC 1A06182	NR_157733.1
2	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Paenibacillus*	80%	1426	97%	99.21%	*Paenibacillus odorifer*, strain DSM 15391	NR_028887.1
3	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	80%	1422	100%	99.79%	*[Brevibacterium] frigoritolerans strain DSM 8801*	NR_117474.1
4	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	*Corynebacterium*	80%	1393	86%	99%	*Corynebacterium nuruki* S6-4 contig00061	NR_117816.1
5	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	80%	1434	99%	99.72%	*Enterococcus thailandicus strain NBRC 101867*	NR_114015.1
6	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	*Ochrobactrum*	80%	1353	97%	99.55%	*Ochrobactrum haematophilum* strain CCUG 38531	NR_042588.1
7*	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	80%	1439	96%	98.92%	*Enterococcus faecium* strain NBRC 100486	NR_113904.1
8	Bacteria	Firmicutes	Bacilli	Lactobacillales	unclassified_ Lactobacillales	-	80%	1456	93%	93.52%	*Enterococcus pseudoavium* strain NBRC 100491	NR_113907.1
9*	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	80%	1428	99%	99.44%	*Enterococcus faecium* strain NBRC 100486	NR_113904.1
10*	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	80%	1442	92%	98.88%	*Enterococcus faecium* strain NBRC 100486	NR_113904.1
11	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	80%	1435	98%	99.51%	*Enterococcus gilvus* strain NBRC 100696	NR_113932.1
12**	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	80%	1432	99%	99.30%	*Bacillus cereus* ATCC 14579	NR_074540.1

*These isolates were identified as belonging to the same strain specie with a high similarity (>98%) to *Enterococcus faecium* strain NBRC 100486

**Although this bacterial isolate was one of the resistant over the generations it was removed because it has been identified with a high similarity (>99%) as a highly pathogenic bacterium

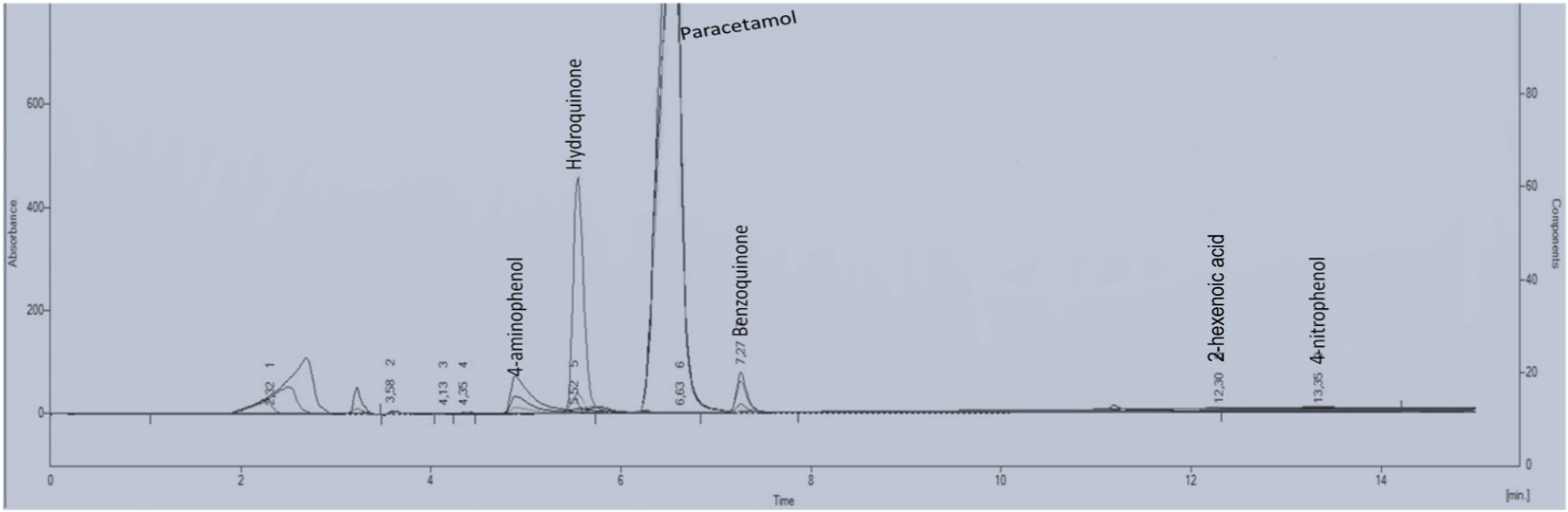


Figure S1 Chromatogram with representative peaks corresponding to paracetamol and its metabolic products 4-aminophenol (4.90 min), hydroquinone (5.52 min), paracetamol (6.63 min), benzoquinone (7.27 min), 2-hexenoic acid (12.30 min) and 4-nitrophenol (13.35)

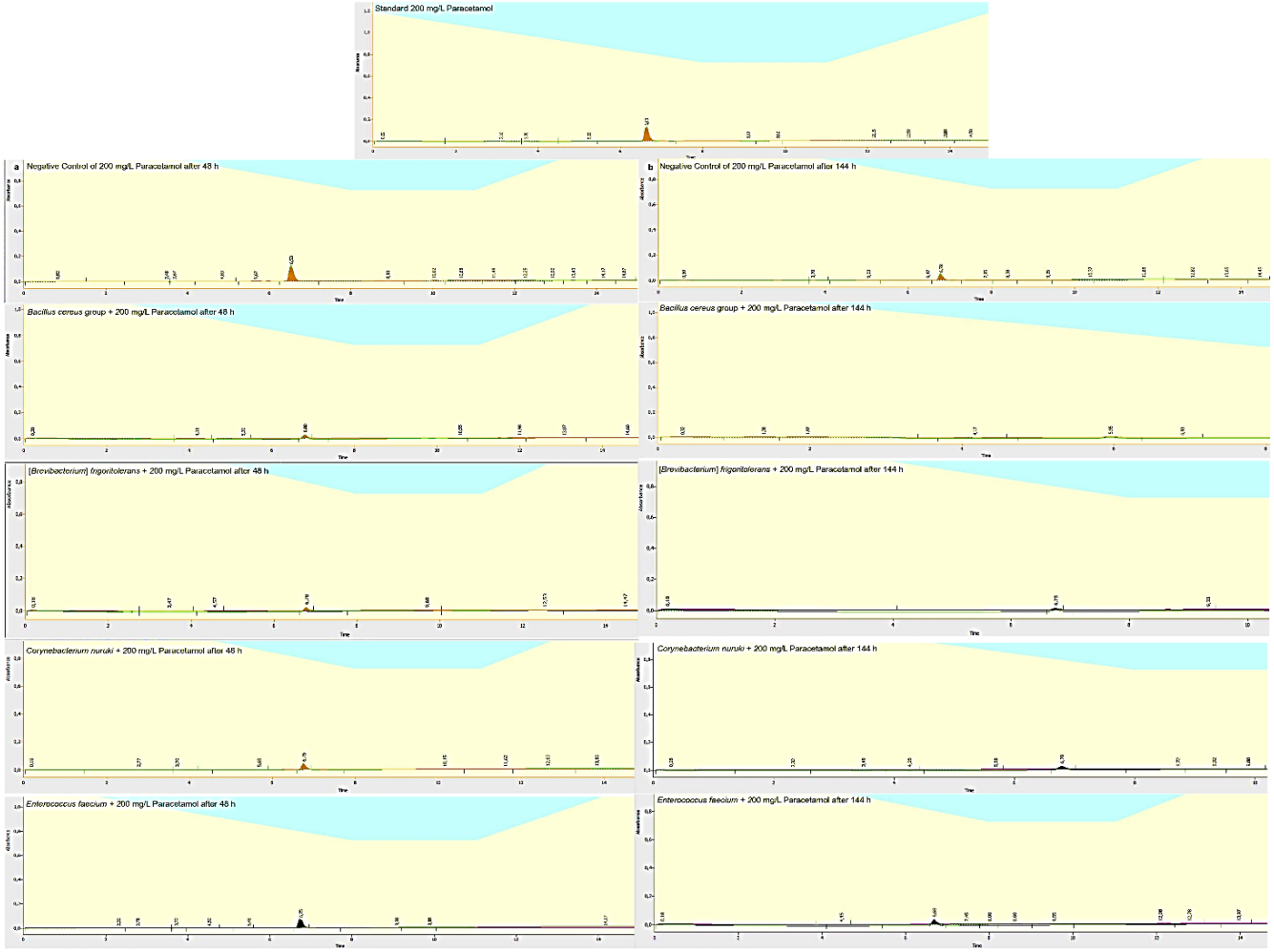


Figure S2 Representative chromatograms corresponding to the analysis of the different isolate cultures (*Bacillus cereus* group, *Corynebacterium nuruki*, *[Brevibacterium] frigoritolerans* and *Enterococcus faecium)* in liquid MSM spiked with 200 mg/L Paracetamol incubated in dark after 48 h (**a**) and144 h (**b**) at 28 ºC

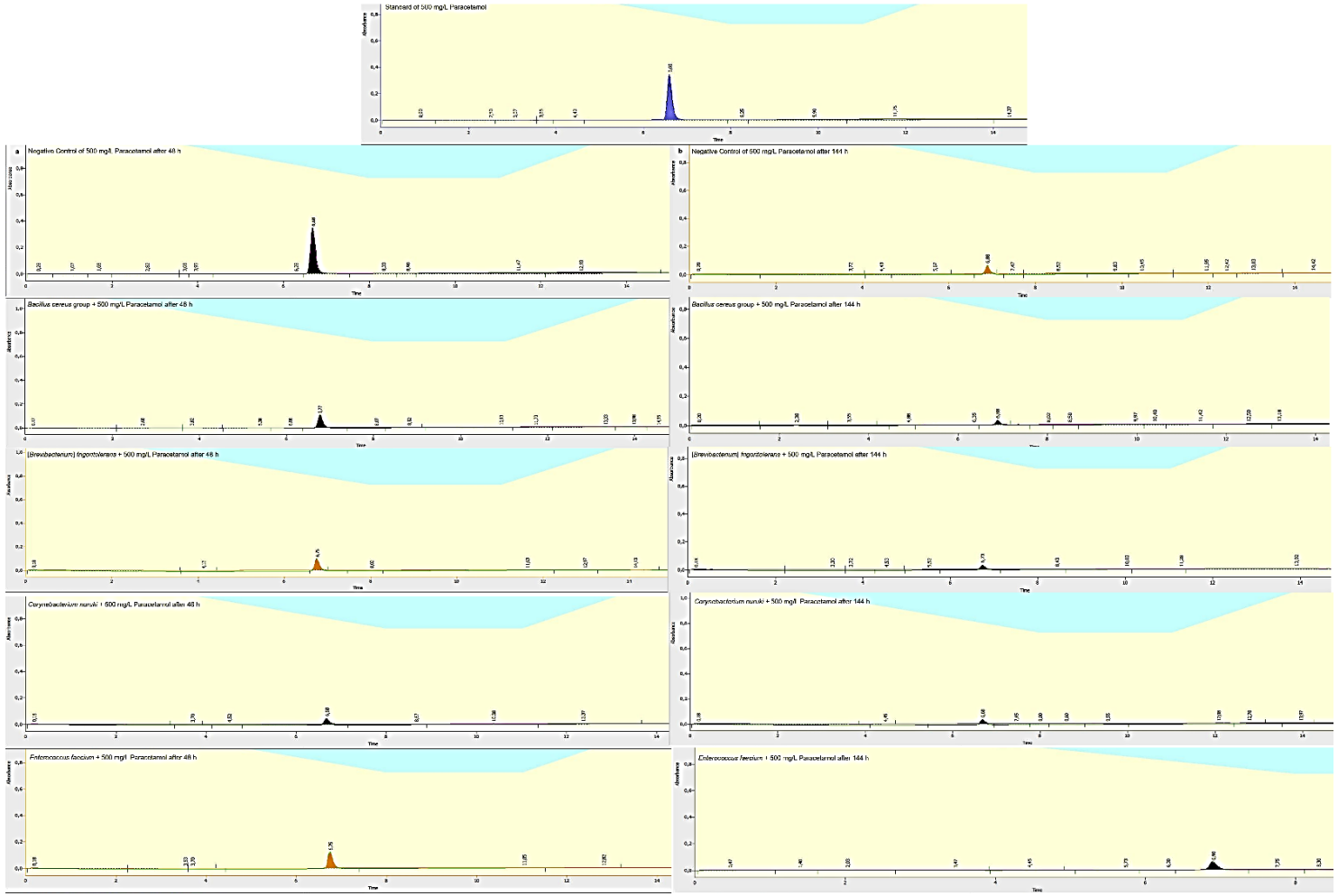


Figure S3 Representative chromatograms corresponding to the analysis of the different isolate cultures (*Bacillus cereus* group, *Corynebacterium nuruki*, *[Brevibacterium] frigoritolerans* and *Enterococcus faecium)* in liquid MSM spiked with 500 mg/L Paracetamol incubated in dark after 48 h (**a**) and144 h (**b**) at 28 ºC

Annex 3 (CHAPTER 5)

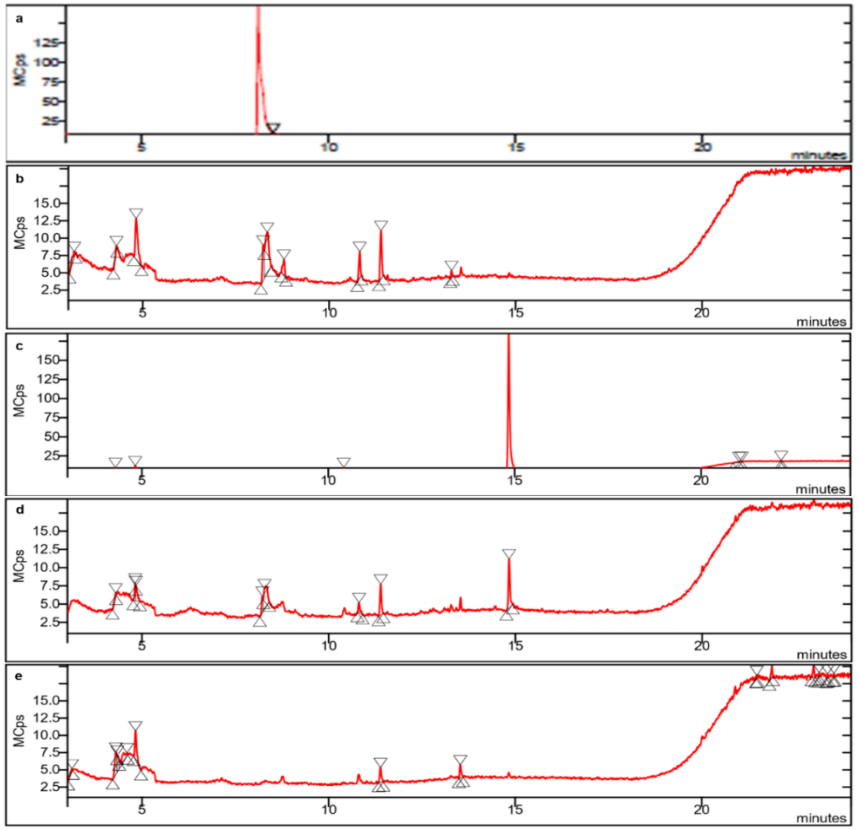


Figure S4 GC-MS chromatograms of FLX's secondary metabolites analysis: FLX standard (**a**), negative control at 50 mg/L FLX (**b**), bacterial consortium cultures in of Postgate B in the presence of 20 mg/L FLX (**c**) and 50 mg/L FLX (**d**) and cultures in MSM at 50 mg/L FLX (**e**) after 28 days of assay

Annex 4 (CHAPTER 6)

Table S5 Sequence contigs (treated data) obtained with the BioEdit software that resulted from DNA Sanger sequencing of each bacterial isolate_FLX

Isolates	Name	BioEdit Contig-0 Sequences
1	*Pseudomonas putida*	ATAGAGTTGGAACTCGAGCCAGAAAGACAAGAGCTTGCTCTTAGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGA CAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTA GCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCT ACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTA AGTTGGGAGGAAGGGCATTAACCTAATACGTTAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACA GAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGC ATCCAAAACTGGCAAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAAATATAGGAAAGGAACACCAGTGGCGAA GGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAA CTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGGTTAAAACTCAAATGAATT GACGGGGGCCCGCACAAGCGGTGGAAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGAT GGATTGGTGCCTTCGGGAACTCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCT TGTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCT TACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGTAGTCCGGATC GCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT CACACCATGGGAGTGGGTTGCTCCAGAAGTAGCTAGTTTAACCTGTCGGGAGGAACGGTTACCCT
2	*Enterobacter ludwigii*	TGCAAGTCGGGCGGTAACACGGGGGAGCTTGCTCCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAA CTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCT AGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACG GGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGC GGGGAGGAAGGTGTTGTGGTTAATAACCACAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCCAGCAGCCGCGGTAATACGG AGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCA TTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGG CGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTT GGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACG GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTAGCAGAGATGCTT TGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATC CTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGA GTAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAG TCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA CCATGGGAGTGGGTTGCTAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCG

3	*Pseudomonas nitritireducens*	CGAGCGGATGATCGGTGAAGCTTGCTCCCAGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAA GGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGG GTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGC AGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAA GGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGC GTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGGTAAGATGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCATAACTGC CTGACTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGG ACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGAAT CCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCA CAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCCGGAATCTTGCAGAGATGCGAGAGTGCCTTCG GGAATCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCA GCACGTTAAGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTA CACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTC GACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAG TGGGTTGCTCACAGAAGTATGCCTAGTCTAACCGCAAGGGGGACGGTTACCAG
4	*Alcaligenes faecalis*	GAACGGCAGCTGCGTAGAGAGCTTAGCTATCTTGGCGGCGAGTGGCGGACGGGTGAGTAATATATCGGAACGTGCCCAGTAGCGGGGGATAACTACT CGAAAGAGTGGCTAATACCGCATACGCCCTACGGGGGAAAGGGGGGGATCGCAAGACCTCTCACTATTGGAGCGGCCGATATCGGATTAGCTAGTTG GTGGGGTAAAGGCTCACCAAGGCAACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAG GCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATCCCGCGTGTATGATGAAGGCCTTCGGGTTGTAAAGTACTTTTGGCAGAG AAGAAAAGGTACCTCCTAATACGAGGTACTGCTGACGGTATCTGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTG CAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGTGTAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGGGCTCAACCTTGGAACTGCATTTTTA ACTGCCGAGCTAGAGTATGTCAGAGGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGAAATATGTGGAGGAATACCGATGGCGAAGGCGCCCC CCTGGGATAATACTGACGCTCAGACACAAAGCGTGGGGAGCAAACAGGATTAATACCCTGGTAGTCCACGCCCTA
5	*Pseudomonas aeruginosa*	TTTGATCTCGGCTCAGAAGTCGGTGAAGCTTGTTCCCGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACG TCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAG TTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGG AGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTG GGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGG GTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCC AAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGA CCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCC GTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGG GCCCGCACAAGCGGTGGAGCATGTGGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGG TGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTT AGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCA GGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTG

		CAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAT GGGAGTGGGTTGCTACAGAAGTAGCCTAGTCTAACAACAAGGGGCAAGGTAAC
7	*Pseudomonas nitroreducens*	GCATAGAGTTGGATCTCGAGCGGATGATGGGAGCTTGCTCCCGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGA CAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTA GCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCT ACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTA AGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACG AAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGGTAAGATGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGC ATCCATAACTGCCTGACTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGG TCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACT AGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGA CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAAGAACCTTACCTTGGCCTTGACATGTCCGGAACTTTGCAGAGATG TGAGGGTGCCTTTGGGAATCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTT GTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCTGGTGACAAACCGGAGGAAGGTGGGGATCGCGTCAAGTCATCATGGCCCTT ACGTCCAGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCG CAGTCTGCAATTTGACTGCGTGAAGTCGGAATCGTTAGTAATCGTGAATCAGAATGTCACGGTGATTACTTTTCTGTTCTTTGTACACACTGCCCGTCA CACCATGGGAGTGGGTTGCTCCAGATGTAGCTAGTCTAACCACAAGGGGCAAGGTTACCCAAT

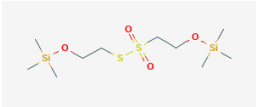
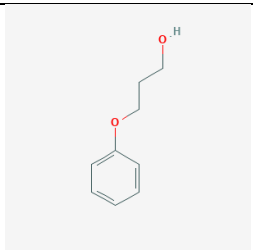
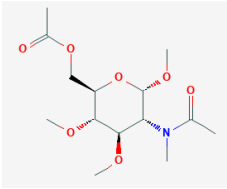
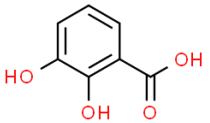
Annex 5 (CHAPTER 7)

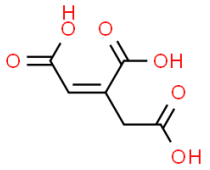
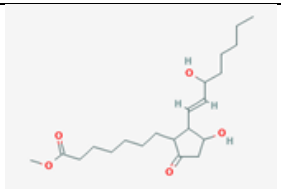
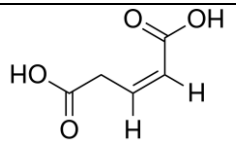
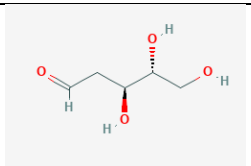
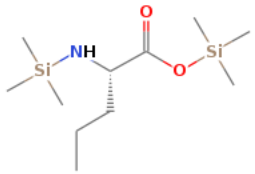
Table S6 Sequence contigs (treated data) obtained with the BioEdit software that resulted from DNA Sanger sequencing of each bacterial isolate_EE2

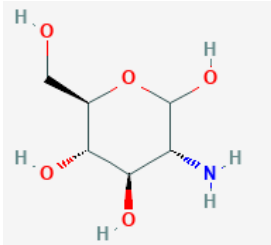
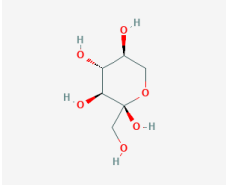
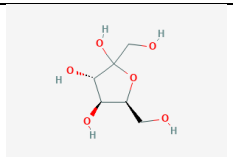
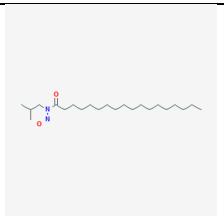
Isolate		BioEdit Contig Sequences
1	*Acinetobacter bouvetii*	>Contig-0 TGCAAGTCGAGCGGAGTTGTGGTGCTTGCACCATAACTTAGCGGCGGACGGGTGAGTAATGCTTAGGAATCTGCCTATTAGTGGGGGACAACGTTTCGAAAGGAACGC TAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGATGAGCCTAAGTCAGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGC GACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGGAAC CCTGATCCAGCCATGCCGCGTGTGTGAAGAAGCCCTTTTGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTCTTCTAGTTAATACCTAGGATGAGTGGACGTTACTCGC AGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTACGTAGGCGGCTTTTTAAGTCG GATGTGAAATCCCTGAGCTTAACTTAGGAATTGCATTCGATACTGGGAAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATC TGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAA ACGATGTCTACTAGCCGTTGGGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGA CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATAGTAAGAACTTTCCAGAGATGGATTGGTGCCTTC GGGAACTTACATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTTCCTTATTTGCCAGCGGGTTAAG CCGGGAACTTTAAGGATACTGCCAGTGACAAACTGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGTCGGT ACAAAGGGTTGCTACCTAGCGATAGGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCG GATCAGAATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAATTTGTTGCACCAGAAGTAGGTAGTCTAACCGCAAGGAGGACGCT T
2	*Acinetobacter kookii*	>Contig-0 TTAGAGTTTGATCTCGGCTCGAAGTCGTTGCTTCGGTAACAGACCTAGCGGCGGACGGGTGAATAATGCTTATGAATCTGCCTATTAGTGGGGGACAACATCTCGAAAG GGATGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTAATATATGAGCCTAAGTCGGATTAACTAGTTGGTGGGGTAAAGGCCTAC CAAGGCGACGATCTGTAGCGGGTCTGAAAGGATGATCCGCCACCCTGGGACTGACACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGG GGGAACCCTGATCCACCCATGCCGCGTGTGTGAATAAGGCCTTTTGGTTGTAAAGCACTTTAAACGAGGAGGAGGCTACTGGTATTAATACTACCGGATAGTGGACGTT ACTCGCAGAATAATCACCGGCTAACTCTGTGCCAACAGCCGCGGTAATACACAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTATGCGGCTTTTTA AGTCGGATGTGAAATCCCTGAGCTTAACTTAGGAATTGCATTCGATACTGGGAGGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAC AGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGC CGTAAACGATGTCTACTAGCCGTTGGGGCCTTTGAGACTTTAGTGGCGCAGATAACGCGATAAGTACACCGCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAATGA ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTTACATACAGAGAACTTTCCAGAGATGGATTGGTG CCTTTGGGAACTCTGATACAGGTGCTTCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTTCCTTACTTGCCATCGGGT CATGCCGGGAATTTTAAGGATACTGCCAGTGACAAACTGGAGGAAGGCGGGGACGACGTCAAGTCACAATGGCCCTTACGACCATGGCTACACACGTGCTACAATGGT CGGTACAAAGGGTTGCTACCTAGCGATAGGATGTTAATTTCAAAAATCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGATTCCATGAAGTTGGAATCGTTAGTAATC GCGGATCAGAATGCCGCGGTGAATACGTTCCCGGGCTTTGTACACACCGCCCGTCACACCATGGGAGTTTGTTGCTCCAGAAGTAGGTTGCTAACCGCAAGTAACAAG GTAACCCT
3	*Pantoea agglomerans*	>Contig-0 ACTTGCAAGTCGGGCGGTAACACGGGTGCTTGTGCTCCCGGTTGACGACCGGCGGATGGGTGATGATTGTCAGGGAGCCTGCTTGAGGGAGGGGGATATCTACTGGGA ACGGTAACTAATACCGCATACCATCGCAGAACCCAAGAGAGCTACCTTCCTTCCTCTTGCCATCGAATGTGCCCCGATGTAATTAGCTGGTGGGTGGGGTAACGGCTCA CCTAAGCAACTATCCCTAGCTGGTCTGAAAGGATGACCACCCTCACTGTAACTGACACCCCGTCCTCACTCCTACGGGAGGCATGGGTGGGAAATATTGCAGAATGGG CGCCTGCCTGATGCATGCATGCCGCGTGTATGAAGAATGCCTTCTGTTTGTAAATTACTTTCACGAGGAAGGAACTTGTTGTGATTAATAATCACGACTAGTGGACGTT

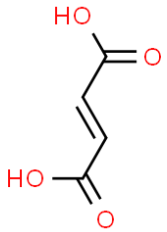
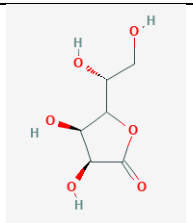
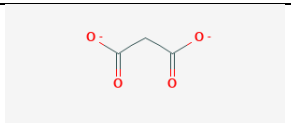
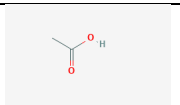
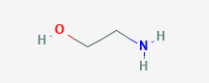
		ACTACCCAGAATAAGAACCGGCTAACTCCGTGCCAGCCAGCCGCGGTAAAATACAGAGGGTGCAAGCGTTAATCGGATTTTTACTGGGCGTAAAGCGCACACGCAGGC GGTTTTTCAAGTCAGATGTGAAATCCCCGAGCTCAACCTGAGAACTGCATTCGATACGTGGCAAGCTAGAATATTGGTAGAAGGATGGTAAAATTCCAGTGTGTAGCG GAGAAATGCATAGAGATCTGGAAGAATACCGATGGCGAACGCCCCCCTCGTGGACAAACACTGACGCTCAGTGTGCGAACAGCGTGGGAGAACAAACAGGATAATAG ATACCCTGGGTAGTCCACGCCGTAAACGATGTCTACTATTGCGACGTTGGGCCCTTGAGGCGTGGCTGTCCGCAGCTAAACGCGATAAGTACAACCGCCTGGGGGGAG TACGGTCGTCAGAGGTAAAACACTCAAATGAATTTGACGGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTAGTCTTG ACATCCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAA CGAGCGCAACCCTTTTCCTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC TTACGAGTAGGGCTACACACGTGCTACAATGGTGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCA ACTCGACTCCATGAAGTCGGAATCGTTAGTATTCGTAGATCAGAAGCCTACGGTGAATACGTTCCCGGCCCTGGTCCCCCCCCCCCTCCCCCCCATGGGATTGGGTTCC ACAGAAATTAGTTACCTAAACCCTAGGGAGGGCGTT
4	*Shinella zoogloeoides*	>Contig-0 TAGAGTTTGATCCTGGCTCAGAAGTCGTAACAAGGTAACCAGTTAGAGTTTGATCCTGGCTCCGAAGCCGTTCCACGGAAAACATCAGGAAAACTTGTGCGAATACCG TATACGCCCTTCGGGGGAAAAATTTATCGGAGTTGGATGAGCCCCCGTTGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAACTGGTCTGAG AGGATGATCACCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCACCCATGCCGCGTG AGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTCACCGGTGAAGATAATGACGGTAACCGGAGAAAAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAA AGGGGCTAGCGTTGTTCGGAATCACTGGGCGTAAAGCGCACGTAGGCGGGTATTTAAGTCAGGGGTGAAATCCCAGAGCTCAACTCTGGAACTGCCTTTGATAATGGG TACCTACAGTATGGAAGAGGTAAGTGGAATTCCTGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCACTGGCGAAGGCGGGCTAACTGGTCCCATTACTG ACGCTGAGGTGCGAAAACGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACCCCGTAAACAATGAATGTGACCCGCCGGCAGGCAGGCATGGTCGGAGGCGC AGCTAACGCATTAAACATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGC AACGCGCAGAACCTTACCAGCCCTTGACATGTCGGTCGCGGATTACAGAGATGTTTTCCTTCAGTTAGGCTGGACCGAACACAGGTGCTGCATGGCTGTCGTCAGCTCG TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGCCGAGAGGAA GGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGTGGTGACAGTGGGCAGCGAGACAGCGATGTCGAGCTAATCTCCAAA AGCCATCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCTGGCCTTGTACACA CCGCCCGTCACACCATCAGAGTAGCCTTTTAGAGTTTGATCCTGGCTCAGAAGTCGTAACAAGGTAACCTG

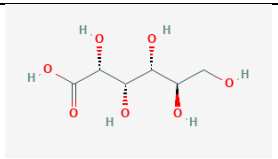
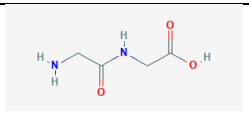
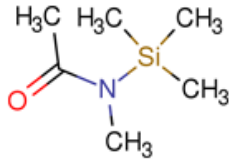
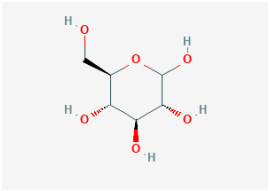
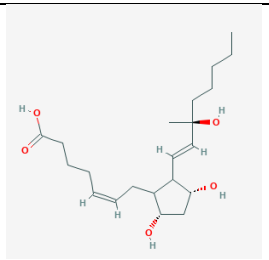
Table S7 GC-MS of the TMS derivatives of metabolic products and its molecular structures produced during the degradation of EE2

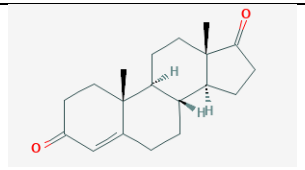
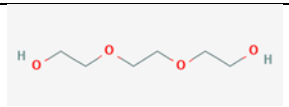
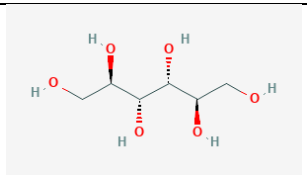
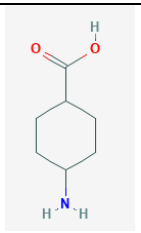
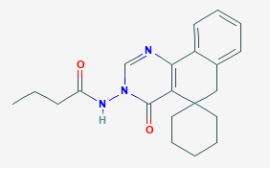
Peaks: RT (min)	Result	Entry #	TMS derivatives of metabolites	Metabolites	Chemical structure
5.542	882	476279	Bis(2-trimethylsiloxyethylthio) disulfide	3,10-Dioxa-6,7-dithia-2,11-disiladodecan	
6.562	479	37863	3-Phenoxy-1-propanol, TMS derivative	3-Phenoxy-1-propanol	
6.628	531	90305	*alpha*-D-Glucopyranoside, methyl 2-(acetylamino)-2-deoxy-, 3,4,6-triacetate	*.alpha.*-D-Glucopyranoside, methyl 2-(acetylmethylamino)-2-deoxy-3,4-di-O-methyl-, 6-acetate	
6.806			2,3-Dihydroxybenzoic acid, 3TMS derivative	2,3-Dihydroxybenzoic acid	

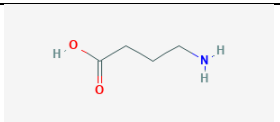
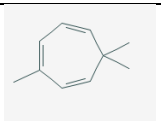
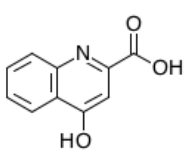
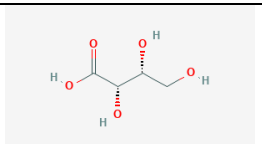
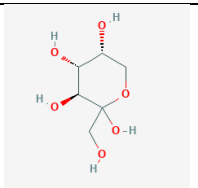
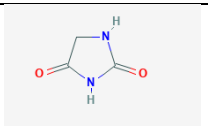
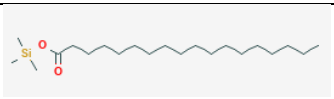
6.874			Aconitic acid, (Z)-, 3TMS derivative	Aconitic acid, (Z)	
6.954	506	393816	Prost-13-en-1-oic acid, 9,11,15-tris[(trimethylsilyl)oxy]-,	Prost-13-en-1-oic acid, 11,15-dihydroxy-9-oxo-, methyl ester, (11*alpha*,13E,15S)-	
7.067	607	401296	2-Pentenedioic acid, bis(trimethylsilyl)	2-Pentenedioic acid or Glutaconic acid	
7.158	564	329318	Erythro-Pentonic acid, 2-deoxy-3,4,5-tris-O-(trimethylsilyl)	Erythro-Pentonic acid or 2-Deoxy-D-ribose	
7.255	641	96212	Norvaline,N,N,O-TMS	Norvaline	

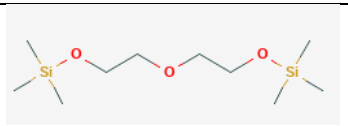
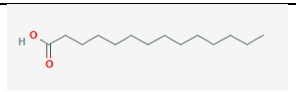
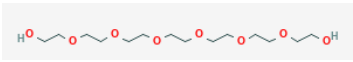
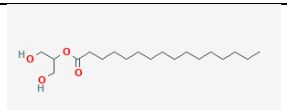
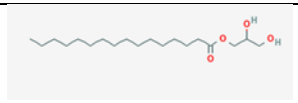
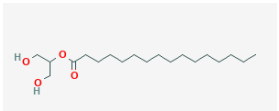
7.444	600	409249	Glucosamine per-TMS	Glucosamine	
7.502	608	55478	L-Sorbopyranose, (1S,2R,3S)-, 5TMS derivative	L-Sorbopyranose	
7.558	787	39493	L-(-)-Sorbofuranose, pentakis(trimethyls	L-(-)-Sorbofuranose,	
7.79	711	164011	Octadecanamide, N-(2-methylpropyl)-N-nitroso- (CAS)	Octadecanamide, N-(2-methylpropyl)-N-nit	

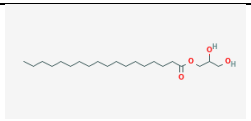
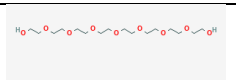
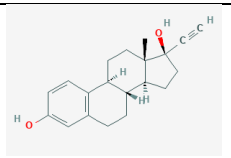
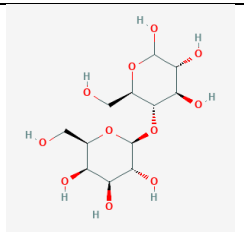
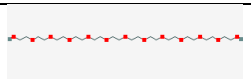
8.099	806	42821	2-Butenedioic acid, (E)-, 2TMS derivative	2-Butenedioic acid or Fumaric acid	
8.59	668	39502	Mannonic acid, .gamma.-lactone, 4TMS derivavative	Mannonic acid 1,4-lactone	
8.692	670	58155		Malonate	
8.697				Acetic acid	
9.018			Allonic acid, .gamma.-lactone, 4TMS derivavative	Allonic acid, *.gamma.*-lactone	
9.192	775	51415		Ethanolamine	

9.268	663	55685		D-Gluconic acid	
9.35	647	143912		Glycylglycine	
9.393	560	171355		Trimethylsilyl 3-{3-chloro-4-[(trimethyl	
9.485	958	371208		Glucopyranose	
9.499	551	37767		Prosta-5,13-dien-1-oic acid	

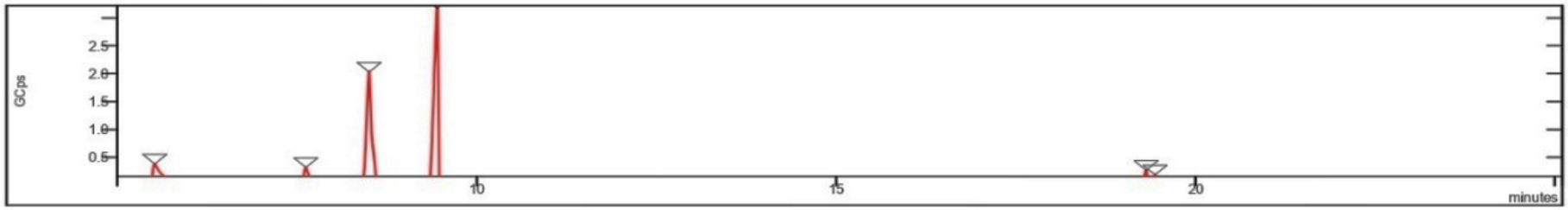
9.571	535	290054		1-Androsten-3,17-dione, di-trimethylsily	
9.905	876	46809		Triethylene glycol,	
9.996	730			Mannitol	
10.158	618	443604		*trans*-4-Aminocyclohexanecarboxylic acid	
10.205	864	83069		Hexadecanoic acid	
10.432	478	58935		1,3-Phenantrolin-4(3H)-one, 5,6-dihydro-3-(1-oxobutylamino)-spiro-5-cyclohexane-	

10.519	586	56120	4-Aminobutanoic acid, 3TMS derivative	*gamma*-Aminobutyric acid	
10.679	655	39165		3,7,7-Trimethylcyclohepta-1,3,5-triene	
11.317	470	394093	2-Quinolinecarboxylic acid, 4-hydroxy-	4-Hydroxy-2-quinolinecarboxylic acid	
11.94	477	359634		Threonic acid	
12.053	684	380151		D-Fructose	
12.133	663	387842		2,4-Imidazolidinedione	
12.188	863	156332		Octadecanoic acid	
12.436	549	245044		6-Amino-1,4-	

				diazathraquinone	
12.491	908	38407		Trimethyl-[2-[2-[2-(2-trimethylsilyloxye	
13.597	818	308778		Myristic acid	
13.815			Edetic Acid, 4TMS derivative		
14.788	471	281272		Pentitol-1,4-d2, 3-desoxy-tetrakis-o-(tr	
15.21	896	86830	Heptaethylene glycol, 2TMS derivative	Heptaethylene glycol	
15.445	703	55082		2-Palmitoylglycerol	
15.915	910	55078		1-Monopalmitin	
16.798	585	261903		(Z)-1-Trimethylsilyl-4-trimethylsilyloxy	
18.591	712	100702		2-Monostearin or 2-Stearoylglycerol	

19.065	921	209278		Glyceryl monostearate	
19.213	902	86831		Octaethylene glycol	
19.315	918	40316	Ethinyl estradiol, 2TMS derivative	Ethinyl estradiol	
19.788			Altronic acid,.gamma.-lactone, 4TMS derivavative		
19.918	884			Lactose	
22.62	811	86856		Dodecaethylene glycol	

Sample ID: 20190611 28 EE Acquisition Date: 11/06/2019 20:17



Unidentified Peaks

RT (min)	Match	Status Result	Quan Ions Type	Library	Area Entry #	Amount/RF Name	Prob.	Match Result	Thresh-old
5,519			RIC		4,178e+9	10,400	77.11	853	300
	1	853	N-F	w10n14m1	476279	3,10-Dioxa-6,7-dithia-2,11-disiladodecan			
	2	818	N-F	mainlib	37707	Bis(2-trimethylsiloxyethylthio)disulfide			
	3	818	N-F	w10n14m1	476280	2,2'-Dithiobisethanol, 2TMS derivative			
6,368			RIC		1,807e+8	0,450	10.35	737	300
	1	737	N-F	mainlib	170816	D-(-)-Ribofuranose, tetrakis(trimethylsi			
	2	737	N-F	w10n14m2	298922	D-(-)-Ribofuranose, tetrakis(trimethylsilyl) ether (isomer 1)			
	3	730	N-F	mainlib	170842	D-Xylofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)-			
6,539			RIC		8,101e+7	0,202	12.44	715	300
	1	715	N-F	mainlib	39502	Mannonic acid, 2,3,5,6-tetrakis-O-(trime			
	2	715	N-F	w10n14m2	327499	Mannonic acid, .gamma.-lactone, 4TMS derivavative			
	3	708	N-F	mainlib	39496	Altronic acid, 2,3,5,6-tetrakis-O-(trimethylsilyl)-, lactone			
6,947			RIC		5,940e+7	0,148	49.50	842	300
	1	842	N-F	mainlib	170841	Arabinofuranose, 1,2,3,5-tetrakis-O-(tri			
	2	842	N-F	w10n14m2	298925	Arabinofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)-			
	3	797	N-F	mainlib	170823	D-Glycero-L-manno-Heptonic acid, 2,3,5,6,7-pentakis-O-(trimethylsilyl)-, .gamma.-lactone			
7,063			RIC		4,921e+7	0,123	36.02	840	300
	1	840	N-F	mainlib	170823	D-Glycero-L-manno-Heptonic acid, 2,3,5,6			
	2	840	N-F	w10n14m2	379818	Glucoheptonolactone, 5TMS derivative			
	3	823	N-F	mainlib	170841	Arabinofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)-			
7,161			RIC		2,134e+7	0,053	52.46	812	300
	1	812	N-F	mainlib	170823	D-Glycero-L-manno-Heptonic acid, 2,3,5,6			
	2	812	N-F	w10n14m2	379818	Glucoheptonolactone, 5TMS derivative			
	3	787	N-F	mainlib	39510	D-Glycero-D-gulo-Heptonic acid, 2,3,5,6,7-pentakis-O-(trimethylsilyl)-, .gamma.-lactone			
7,244			RIC		2,836e+7	0,071	29.26	723	300
	1	723	N-F	w10n14r	55523	TRIMETHYLSILYL 1,2,3,4-TETRAKIS-O-(TRIME			
	2	707	N-F	w10n14m2	375734	TRIMETHYLSILYL 2,3,4,5-TETRAKIS-O-(TRIMETHYLSILYL)HEXURONATE			
	3	700	N-F	w10n14m2	375696	TRIMETHYLSILYL 1,2,3,4-TETRAKIS-O-(TRIMETHYLSILYL)HEXOPYRANURONATE			
7,477			RIC		1,488e+8	0,370	8.80	900	300
	1	900	N-F	mainlib	39528	D-Arabinose, tetrakis(trimethylsilyl)-			
	2	899	N-F	w10n14m2	298933	1,2,3,4-TETRAKIS-O-(TRIMETHYLSILYL)PENTOPYRANOSE			
	3	899	N-F	w10n14m2	298944	D-Arabinose, 4TMS derivative			
7,545			RIC		4,314e+8	1,074	22.32	865	300

	1	865	N-F	mainlib	39490	D-(-)-Tagatofuranose, pentakis(trimethyl
	2	865	N-F	wl0nl4m2	371230	D-(-)-Tagatofuranose, pentakis(trimethylsilyl) ether (isomer 1)
	3	860	N-F	mainlib	170818	D-(-)-Tagatofuranose, pentakis(trimethylsilyl) ether (isomer 2)
7,622			RIC		1,905e+9	4,742 16.18 882 300
	1	882	N-F	mainlib	39369	D-Psicopyranose, pentakis(trimethylsilyl
	2	882	N-F	mainlib	39373	D-(-)-Fructopyranose, pentakis(trimethylsilyl) ether (isomer 2)
	3	882	N-F	wl0nl4m2	371243	D-Psicopyranose, 5TMS derivative (isomer 2)
7,805			RIC		1,197e+8	0,298 25.18 789 300
	1	789	N-F	mainlib	164009	Glucosamine per-TMS
	2	789	N-F	wl0nl4m2	389358	D-(+)-Glucosamine, 6TMS derivative
	3	789	N-F	wl0nl4m2	389359	Glucosamine per-TMS
7,896			RIC		5,653e+8	1,407 32.40 873 300
	1	873	N-F	mainlib	170819	D-(+)-Talofuranose, pentakis(trimethylsi
	2	873	N-F	wl0nl4m2	371182	D-(+)-Talofuranose, pentakis(trimethylsilyl) ether (isomer 2)
	3	867	N-F	mainlib	170820	D-(+)-Talofuranose, pentakis(trimethylsilyl) ether (isomer 1)
8,172			RIC		1,805e+8	0,449 13.67 690 300
	1	690	N-F	wl0nl4r	55482	D-Fructose, 5TMS derivative
	2	686	N-F	mainlib	39490	D-(-)-Tagatofuranose, pentakis(trimethylsilyl) ether (isomer 1)
	3	686	N-F	wl0nl4m2	371230	D-(-)-Tagatofuranose, pentakis(trimethylsilyl) ether (isomer 1)
8,327			RIC		1,865e+8	0,464 30.90 689 300
	1	689	N-F	wl0nl4m2	375695	TRIMETHYLSILYL 1,2,3,4-TETRAKIS-O-(TRIME
	2	687	N-F	mainlib	38850	2-Keto-d-gluconic acid, pentakis(O-trimethylsilyl)-
	3	687	N-F	wl0nl4m2	375697	2-KETO-D-GLUCONIC ACID 5TMS
8,503			RIC		1,129e+10	28,108 8.79 967 300
	1	967	N-F	mainlib	39377	Glucopyranose, pentakis-O-trimethylsilyl
	2	967	N-F	wl0nl4m2	371208	Glucopyranose, 5TMS derivative
	3	957	N-F	wl0nl4r	55460	D-Mannopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-
8,675			RIC		2,444e+8	0,608 33.63 727 300
	1	727	N-F	wl0nl4m2	12923	2-[(Trimethylsilylmethylamino)methylthi
	2	698	N-F	wl0nl4r	55644	Myo-Inositol, 6TMS derivative
	3	695	N-F	wl0nl4m2	58163	2,3-di(trimethylsiloxy)-3-(trimethylsiloxycarbonyl)-2-propanoic acid
8,734			RIC		3,407e+8	0,848 37.89 822 300
	1	822	N-F	mainlib	164010	D-Galactose, 2-deoxy-3,4,5,6-tetrakis-O-
	2	822	N-F	wl0nl4m2	370776	2-Amino-2-deoxyhexose, 5TMS derivative
	3	812	N-F	mainlib	164009	Glucosamine per-TMS
8,801			RIC		2,415e+8	0,601 28.89 721 300
	1	721	N-F	mainlib	164011	Octadecanamide, N-(2-methylpropyl)-N-nit
	2	721	N-F	wl0nl4m2	183198	Octadecanamide, N-(2-methylpropyl)-N-nitroso- (CAS)
	3	721	N-F	wl0nl4m2	183199	Octadecanamide, N-(2-methylpropyl)-N-nitroso-
8,942			RIC		6,291e+7	0,157 8.96 758 300
	1	758	N-F	wl0nl4r	55458	Gulose, 2,3,4,5,6-pentakis-O-(trimethyls
	2	755	N-F	wl0nl4r	54615	D-Xylopyranose, 1,2,3,4-tetrakis-O-(trimethylsilyl)-
	3	749	N-F	wl0nl4r	55163	.alpha.-D-Glucopyranoside, methyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-
9,087			RIC		2,223e+8	0,553 43.67 943 300
	1	943	N-F	mainlib	164009	Glucosamine per-TMS
	2	943	N-F	wl0nl4m2	389358	D-(+)-Glucosamine, 6TMS derivative
	3	943	N-F	wl0nl4m2	389359	Glucosamine per-TMS
9,449			RIC		1,297e+10	32,289 6.77 958 300
	1	958	N-F	mainlib	39377	Glucopyranose, pentakis-O-trimethylsilyl
	2	958	N-F	wl0nl4m2	371208	Glucopyranose, 5TMS derivative
	3	956	N-F	mainlib	164533	.beta.-D-(+)-Mannopyranose, pentakis(trimethylsilyl) ether
9,488			RIC		2,686e+7	0,067 6.46 946 300
	1	946	N-F	mainlib	39377	Glucopyranose, pentakis-O-trimethylsilyl
	2	946	N-F	wl0nl4m2	371208	Glucopyranose, 5TMS derivative
	3	944	N-F	wl0nl4r	55471	.beta.-D-Glucopyranose, 5TMS derivative

RT (min)	Match	Status Result	Quan Ions Type	Library	Area Entry #	Amount/RF	Prob.	Match Result	Thresh-old	Name
9,542			RIC		3,239e+8		0,806	5.77	827	300	
	1	827	N-F	w10n14r	55458						Gulose, 2,3,4,5,6-pentakis-O-(trimethyls
	2	827	N-F	w10n14r	55471						.beta.-D-Glucopyranose, 5TMS derivative
	3	827	N-F	w10n14m2	371223						.beta.-D-Glucopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)- (CAS)
9,638			RIC		3,399e+6		0,008	22.17	769	300	
	1	769	N-F	w10n14r	55685						D-Gluconic acid, 6TMS derivative
	2	769	N-F	w10n14m2	392114						D-GLUCONIC ACID 6TMS
	3	755	N-F	w10n14m2	375734						TRIMETHYLSILYL 2,3,4,5-TETRAKIS-O-(TRIMETHYLSILYL)HEXURONATE
9,761			RIC		1,221e+7		0,030	8.97	712	300	
	1	712	N-F	w10n14r	55563						D-Fructose, 1,3,4,5,6-pentakis-O-(trimet
	2	711	N-F	w10n14m2	380151						D-FRUCTOSE, O-METHYLOXIM, PENTAKIS-O-(TRIMETHYLSILYL)-
	3	711	N-F	w10n14m2	380152						D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime
9,902			RIC		1,107e+7		0,028	26.44	802	300	
	1	802	N-F	w10n14r	46808						Triethylene glycol, 2TMS derivative
	2	802	N-F	w10n14m1	465303						2,2,13,13-TETRAMETHYL-3,6,9,12-TETRAOXA-2,13-DISILATETRADECANE
	3	802	N-F	w10n14m1	465307						3,6,9,12-Tetraoxa-2,13-disilatetradecane, 2,2,13,13-tetramethyl- (CAS)
9,992			RIC		1,443e+7		0,036	12.59	787	300	
	1	787	N-F	w10n14m2	380133						(3E,5S,6R,7S,8R)-11,11-dimethyl-5,6,7,8-
	2	778	N-F	w10n14r	55560						d-Galactose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme, (1Z)-
	3	774	N-F	w10n14r	55557						d-Galactose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme, (1E)-
10,207			RIC		2,883e+7		0,072	85.39	820	300	
	1	820	N-F	w10n14m2	83069						Hexadecanoic acid, trimethylsilyl ester
	2	810	N-F	w10n14r	50036						Palmitic Acid, TMS derivative
	3	807	N-F	w10n14r	50037						Hexadecanoic acid, trimethylsilyl ester
10,213			RIC		4,095e+7		0,102	85.39	820	300	
	1	820	N-F	w10n14m2	83069						Hexadecanoic acid, trimethylsilyl ester
	2	810	N-F	w10n14r	50036						Palmitic Acid, TMS derivative
	3	807	N-F	w10n14r	50037						Hexadecanoic acid, trimethylsilyl ester
10,428			RIC		9,036e+6		0,022	35.62	550	300	
	1	550	N-F	mainlib	38495						(Z)-Trimethylsilyl 7-((2R,3R,4S)-4,6-bis
	2	550	N-F	w10n14m2	396006						(Z)-Trimethylsilyl 7-((2R,3R,4S)-4,6-bis(trimethylsilyloxy)-2-[(S,E)-3-(trimethylsilyloxy)oct-
	3	521	N-F	mainlib	37726						Prosta-5,13-dien-1-oic acid, 9,11,15-tris[(trimethylsilyl)oxy]-, trimethylsilyl ester, (5Z,9.b
10,510			RIC		7,252e+6		0,018	18.38	543	300	
	1	543	N-F	mainlib	37726						Prosta-5,13-dien-1-oic acid, 9,11,15-tri
	2	543	N-F	w10n14m2	394106						(+)-Prostaglandin F2.beta., 4TMS derivative
	3	541	N-F	mainlib	39267						2,4-Imidazolidinedione, 5-[3,4-bis[(trimethylsilyl)oxy]phenyl]-3-methyl-5-phenyl-1-(trimethyls
10,671			RIC		1,082e+7		0,027	70.31	661	300	
	1	661	N-F	mainlib	39165						1,3,5-Cycloheptatriene, 7,7-dimethyl-3-(
	2	661	N-F	w10n14m1	148683						1,3,5-Cycloheptatriene, 7,7-dimethyl-3-(trimethylsilyl)-
	3	588	N-F	w10n14m1	110112						2(1H)-Isoquinolinecarbothioaldehyde, 3,4-dihydro-
10,782			RIC		5,650e+6		0,014	34.85	708	300	
	1	708	N-F	w10n14m2	93225						3,7-Dioxa-2,8-disilanonane, 2,2,8,8-tetr
	2	692	N-F	w10n14m2	409194						OXALACETATE, TBS 2X, METHOXIM
	3	681	N-F	w10n14r	54958						Ascorbic acid, 4TMS derivative
10,933			RIC		1,318e+8		0,328	13.50	906	300	
	1	906	N-F	mainlib	143916						N-.alpha.-Acetyl-L-Lysine, N,N-trimethyl
	2	906	N-F	w10n14m2	251744						N-.alpha.-Acetyl-L-Lysine, 3TMS derivative
	3	905	N-F	w10n14m2	366992						2-(3',4',6-TRIS(TRIMETHYLSILYLOXY)PHENYL)ETHYL-N,N-BIS(TRIMETHYLSILYL)AMINE
11,392			RIC		7,427e+6		0,018	39.56	756	300	
	1	756	N-F	w10n14m2	375695						TRIMETHYLSILYL 1,2,3,4-TETRAKIS-O-(TRIME
	2	754	N-F	mainlib	38850						2-Keto-d-gluconic acid, pentakis(O-trimethylsilyl)-
	3	754	N-F	w10n14m2	375697						2-KETO-D-GLUCONIC ACID 5TMS

RT (min)	Match	Status Result	Quan Ions Type	Library	Area Entry #	Amount/RF Prob.	Match Result	Thresh-old Name
11,903			RIC		1,355e+7	0,034 29.21	624	300
	1	624	N-F	mainlib	39071			Trimethylsilyl 2-(trimethylsilyloxy)prop
	2	624	N-F	w10n14m1	323600			Trimethylsilyl 2-(trimethylsilyloxy)propaneperoxoate
	3	614	N-F	mainlib	39067			1,4-Cyclohexadiene, 6-isopropenyl-2,4-dimethyl-1,3-bis(trimethylsilyl)-
11,990			RIC		4,919e+7	0,122 21.17	819	300
	1	819	N-F	mainlib	38943			D-(+)-Turanose, octakis(trimethylsilyl)
	2	819	N-F	w10n14m2	406976			D-(+)-Turanose, octakis(trimethylsilyl) ether
	3	812	N-F	w10n14m2	399857			6,7-DIHYDROXYCOUMARIN-.beta.-D-GLUCOPYRANOSIDE, PENTA-TMS
12,045			RIC		7,879e+6	0,020 6.93	704	300
	1	704	N-F	mainlib	170823			D-Glycero-L-manno-Heptonic acid, 2,3,5,6
	2	704	N-F	w10n14m2	379818			Glucoheptonolactone, 5TMS derivative
	3	704	N-F	mainlib	170816			D-(-)-Ribofuranose, tetrakis(trimethylsilyl) ether (isomer 1)
12,183			RIC		4,374e+7	0,109 77.00	841	300
	1	841	N-F	mainlib	86865			Octadecanoic acid, trimethylsilyl ester
	2	841	N-F	w10n14m2	156330			Stearic acid, TMS derivative
	3	840	N-F	w10n14r	51587			Stearic acid, TMS derivative
12,482			RIC		3,679e+7	0,092 17.85	890	300
	1	890	N-F	w10n14r	46809			Triethylene glycol, 2TMS derivative
	2	890	N-F	w10n14m1	465304			TRIETHYLENE GLYCOL, DI-TMS
	3	890	N-F	w10n14m1	465306			3,6,9,12-Tetraoxa-2,13-disilatetradecane, 2,2,13,13-tetramethyl- (CAS)
15,195			RIC		8,985e+7	0,224 13.63	896	300
	1	896	N-F	mainlib	86830			Trimethyl-[2-[2-[2-[2-[2-[2-(2-trimethyl
	2	896	N-F	w10n14m2	331324			Heptaethylene glycol, 2TMS derivative
	3	890	N-F	mainlib	86829			Trimethyl-[2-[2-[2-[2-[2-(2-trimethylsilyloxyethoxy)ethoxy]ethoxy]ethoxy]ethoxy]silane
15,902			RIC		2,752e+8	0,685 96.98	911	300
	1	911	N-F	w10n14r	55078			1-Monopalmitin, 2TMS derivative
	2	911	N-F	w10n14m2	334951			BIS-O-TRIMETHYLSILYL-PALMITINIC ACID-GLYCERIN-(1)-MONOESTER
	3	909	N-F	mainlib	37037			Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester
16,885			RIC		2,755e+8	0,686 16.80	742	300
	1	742	N-F	mainlib	38271			Deoxyfructosazine, heptakis(trimethylsil
	2	742	N-F	w10n14m2	405055			Deoxyfructosazine, heptakis(trimethylsilyl)- deriv.
	3	717	N-F	w10n14m2	380151			D-FRUCTOSE, O-METHYLOXIM, PENTAKIS-O-(TRIMETHYLSILYL)-
17,440			RIC		1,750e+7	0,044 7.44	598	300
	1	598	N-F	w10n14r	53992			3,8-Dioxa-2,9-disiladecane, 2,2,9,9-tetr
	2	597	N-F	mainlib	38850			2-Keto-d-gluconic acid, pentakis(O-trimethylsilyl)-
	3	597	N-F	w10n14m2	375695			TRIMETHYLSILYL 1,2,3,4-TETRAKIS-O-(TRIMETHYLSILYL)HEXOPYRANURONATE
18,394			RIC		4,939e+7	0,123 34.16	724	300
	1	724	N-F	mainlib	38850			2-Keto-d-gluconic acid, pentakis(O-trime
	2	724	N-F	w10n14m2	375697			2-KETO-D-GLUCONIC ACID 5TMS
	3	724	N-F	w10n14m2	375727			2-Keto-d-gluconic acid, pentakis(O-trimethylsilyl)-
18,617			RIC		2,406e+8	0,599 10.29	679	300
	1	679	N-F	w10n14m2	409241			MALTOSE MEOX2 TMS
	2	675	N-F	mainlib	38726			Lactulose, octakis(trimethylsilyl) ether (isomer 1)
	3	675	N-F	w10n14m2	407015			Lactulose, octakis(trimethylsilyl) ether (isomer 1)
18,951			RIC		6,459e+8	1,608 31.22	750	300
	1	750	N-F	w10n14m2	409241			MALTOSE MEOX2 TMS
	2	715	N-F	mainlib	38726			Lactulose, octakis(trimethylsilyl) ether (isomer 1)
	3	715	N-F	w10n14m2	407015			Lactulose, octakis(trimethylsilyl) ether (isomer 1)
19,057			RIC		7,129e+8	1,775 50.00	807	300
	1	807	N-F	w10n14m2	354329			Glycerol monostearate, 2TMS derivative
	2	804	N-F	mainlib	209278			Octadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester
	3	804	N-F	w10n14r	55285			Octadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester

RT (min)	Match	Status Result	Quan Ions Type	Library	Area Entry #	Amount/RF	Prob.	Match Result	Thresh-old	Name
19,210			RIC		4,440e+8	1,105	11.52	692	300	
	1	692	N-F	w10n14r	46808					Triethylene glycol, 2TMS derivative
	2	692	N-F	w10n14m1	465303					2,2,13,13-TETRAMETHYL-3,6,9,12-TETRAOXA-2,13-DISILATETRADECANE
	3	692	N-F	w10n14m1	465307					3,6,9,12-Tetraoxa-2,13-disilatetradecane, 2,2,13,13-tetramethyl- (CAS)
19,313			RIC		9,786e+8	2,436	79.82	855	300	
	1	855	N-F	mainlib	40316					19-Norpregna-1,3,5(10)-trien-20-yne, 3,1
	2	855	N-F	w10n14m2	301525					Ethinyl estradiol, 2TMS derivative
	3	819	N-F	w10n14m2	301526					1,3,5(10)-Estratrien-17.alpha.-ethynyl-3,17.beta.-diol, di-trimethylsilyl
19,437			RIC		1,447e+9	3,603	5.79	813	300	
	1	813	N-F	mainlib	39385					3-.alpha.-Mannobiose, octakis(trimethyls
	2	813	N-F	w10n14m2	406987					3-.alpha.-Mannobiose, octakis(trimethylsilyl) ether (isomer 1)
	3	811	N-F	mainlib	39365					Lactulose, octakis(trimethylsilyl) ether, methyloxime (isomer 1)
19,753			RIC		8,939e+6	0,022	38.91	510	300	
	1	510	N-F	mainlib	205123					2,4-Diamino-7,8,9,10-tetrahydro-11-(3-py
	2	510	N-F	w10n14m2	134848					2,4-Diamino-7,8,9,10-tetrahydro-11-(3-pyridyl)pyrimido[5',4':2,3]thieno[5,4-b]quinoline
	3	510	N-F	w10n14m2	134849					2,4-Diamino-7,8,9,10-tetrahydro-11-(3-pyridyl)pyrimido[5',4':2,3]thieno[5,4-b]quinoline
19,883			RIC		1,467e+7	0,037	12.01	580	300	
	1	580	N-F	w10n14m2	314288					PER TRIMETHYLSILYL DERIVATIVE OF 1,4-ANH
	2	569	N-F	w10n14r	55685					D-Gluconic acid, 6TMS derivative
	3	569	N-F	w10n14m2	392114					D-GLUCONIC ACID 6TMS
19,922			RIC		2,639e+7	0,066	5.56	692	300	
	1	692	N-F	w10n14m2	407010					MELIBIOSE 8TMS
	2	691	N-F	w10n14r	55849					Melibiose, octakis(trimethylsilyl)-
	3	686	N-F	mainlib	39523					D-Ribose, 2,3,4,5-tetrakis-O-(trimethylsilyl)-
20,098			RIC		9,359e+7	0,233	39.46	618	300	
	1	618	N-F	w10n14m2	398284					N,N'-bis[(Tetrahydropyranoxy)ethoxyethyl
	2	564	N-F	w10n14m2	398298					propanedinitrile, 2-[2,6-bis[-2-[4-(diphenylamino)phenyl]ethenyl]-4H-pyran-4-ylidene]-
	3	548	N-F	w10n14r	55337					Ribitol, 5TMS derivative
20,196			RIC		3,873e+8	0,964	7.14	734	300	
	1	734	N-F	w10n14r	55557					d-Galactose, 2,3,4,5,6-pentakis-O-(trime
	2	734	N-F	mainlib	40087					2-.alpha.-Mannobiose, octakis(trimethylsilyl) ether, methyloxime (isomer 2)
	3	734	N-F	w10n14m2	407287					2-.alpha.-Mannobiose, octakis(trimethylsilyl) ether, methyloxime (isomer 2)
20,507			RIC		2,054e+7	0,051	8.01	591	300	
	1	591	N-F	mainlib	39492					D-Psicofuranose, pentakis(trimethylsilyl
	2	591	N-F	w10n14m2	371232					D-Psicofuranose, pentakis(trimethylsilyl) ether (isomer 2)
	3	578	N-F	mainlib	39490					D-(-)-Tagatofuranose, pentakis(trimethylsilyl) ether (isomer 1)
20,992			RIC		4,930e+6	0,012	8.16	800	300	
	1	800	N-F	w10n14m2	409242					MANNITOL TMS
	2	791	N-F	w10n14m2	380133					(3E,5S,6R,7S,8R)-11,11-dimethyl-5,6,7,8-tetrakis[(trimethylsilyl)oxy]-2,10-dioxa-3-aza-11-sila
	3	777	N-F	w10n14m2	383470					D-(+)-Galactose, pentakis(trimethylsilyl) ether, ethyloxime (isomer 2)
21,705			RIC		9,793e+7	0,244	19.25	891	300	
	1	891	N-F	mainlib	86831					Trimethyl-[2-[2-[2-[2-[2-[2-[2-(2-trimet
	2	891	N-F	w10n14m2	360506					Octaethylene glycol, 2TMS derivative
	3	886	N-F	mainlib	86832					Trimethyl-[2-[2-[2-[2-[2-[2-[2-[2-(2-trimethylsilyloxyethoxy)ethoxy]ethoxy]ethoxy]ethoxy]ethox
24,983			RIC		4,647e+7	0,116	18.70	878	300	
	1	878	N-F	mainlib	86832					Trimethyl-[2-[2-[2-[2-[2-[2-[2-[2-(2-tri
	2	878	N-F	w10n14m2	377002					Nonaethylene glycol, 2TMS derivative
	3	876	N-F	mainlib	86831					Trimethyl-[2-[2-[2-[2-[2-[2-[2-(2-trimethylsilyloxyethoxy)ethoxy]ethoxy]ethoxy]ethoxy]ethoxy]e
					4,017e+10	100,000				

Match Types: N-F : Normal-Forward

Figure S5 Representative GC-MS report of EE2 and its metabolic products analysis during the experiment with the obtained bacterial isolates

About the author

Tânia Cristina da Luz Palma was born 8 June 1982, in Faro, Portugal. In September 2007, she completed her Biotechnological Engineering degree (pre-bologna). She won an Erasmus Grant (from 07-2-2007 to 30-7-2007) that allowed her to develop a thesis about "Potencial interactions between the cationic liposome X and some LPS binding proteins" in the Service de Génétique Appliquée, Institut de Biologie et de Médecine Moléculaire (Université Libre de Bruxelles), Belgium. From 06-11-2008 to 08-5-2009, she worked as engineer in a genetic diversity study of dairy cows in a "spin-off" enterprise, Progenus S.A. (Gembloux, Belgium), under a Leonardo Da Vinci Grant. In April 2010, she started to work as a research fellow in a research project PTDC/AGR-AAM/102664/2008 entitled "Será a espécie *Plantago almogravensis* hiperacumuladora de alumínio? Elucidação dos mecanismos de tolerância usando plantas micropropagadas" financed by Foundation for Science and Technology (FCT), in the Laboratory of Plant Biotecnology, Faculty of Sciences and Technology, University of Algarve. From this research resulted the publication of three papers in International Peer Reviewed Journals. In 2013, she started to work in the Environmental Technology Laboratory (EcoReach Group) at Centro de Ciências do Mar (CCMAR), University of Algarve, under the supervision of Prof. Dr. Maria Clara Costa. Firstly, as research technician in bioremediation and biosynthesis studies for metal removal and/or recovery using chemical and biological approaches, and then moved as research fellow to other project (PTDC/AAG-TEC/2721/2012) entitled "Bionanomine - Bio-síntese de nanomateriais semicondutores usando resíduos de minas e aplicações amigas do ambiente" financed by FCT, which was about the same subject of work of ETL's group above mentioned. From this activity resulted a paper publication in an International Peer Reviewed Journal and a proceeding paper in 10th Iberian and 7th Iberoamerican Congress on Environmental Contamination and Toxicology (CICTA 2015), Vila Real, Portugal. In the same year she obtained the equivalence to a master's degree in Biological Engineering. In June 2014, she iniciated her PhD fellow (SFRH/BD/95075/2013) at the EcoReach Group from CCMAR, University of Algarve, funded by FCT. The results obtained during her PhD fellowship are presented in this thesis.