Universidade do Minho
Escola de Engenharia

Joana Sofia Gomes da Costa

Effect of carbon nanomaterials on the anaerobic treatment of wastewater containing pharmaceuticals

Dissertação de Mestrado

Mestrado em Biotecnologia

Trabalho efetuado sob a orientação de

Doutora Luciana José Ribeiro Pereira

Doutora Andreia Filipa Ferreira Salvador

Outubro de 2018

DECLARAÇÃO

Nome: Joana Sofia Gomes da Costa

Endereço eletrónico: pg32841@alunos.uminho.pt Telefone: 962579106

Bilhete de Identidade/Cartão do Cidadão: 14843245

Título da dissertação: Effect of carbon nanomaterials on the anaerobic treatment of wastewater containing pharmaceuticals.

Orientadores:

Professora Doutora Luciana José Ribeiro Pereira

Professora Doutora Andreia Filipa Ferreira Salvador

Ano de conclusão: 2018

Mestrado em Biotecnologia

É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA DISSERTAÇÃO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

Universidade do Minho, _____/_____/_____

Assinatura:

AGRADECIMENTOS

Este projeto apenas foi possível com a contribuição de várias pessoas, quer pela partilha de conhecimentos, quer pelo apoio oferecido ao longo da dissertação, percurso académico e de toda a vida. A todas essas pessoas o meu muito obrigado!

Agradeço especialmente às minhas orientadoras, Luciana Pereira e Andreia Salvador, por estarem sempre prontas a esclarecer todas as minhas dúvidas e por toda a paciência. À Rita Silva, que me acompanhou ao longo dos últimos meses, enfrentando comigo o desafio das garrafinhas e ao Gilberto pela ajuda no arranque do trabalho.

Aos colegas do LBA e ao staff do CEB. À Carlinha e ao Valdo, por trazerem a alegria com eles cada vez que estavam connosco no laboratório; à Mags, por estar presente em cada segundo, sem dúvida que arrecadamos bastantes aventuras no mundo da engenharia e a ti devo muito do que consegui, principalmente nestes últimos dois anos; ao Mile e ao Filipe por todo o companheirismo e ajuda nos momentos em que o trabalho mais apertava. Não podia ter pedido melhores colegas de laboratório e amigos.

Aos meus colegas de mestrado, principalmente à "team marmitas" por todas as gargalhadas e momentos de terapia emocional. Aos Spoon, principalmente o Pedro que mesmo frente a todas as adversidades sempre arranjou energia para me ajudar com os meus dramas, obrigada por seres o melhor confidente, o melhor amigo e o melhor irmão desta academia. Ao Rogério, por "obrigar" sempre a que me supere, és uma inspiração e espero um dia ser como tu "meu pequeno"!

Aos meus amigos, Manela, Joana, Caroline, "Lindas", Carlinhos, Hélder e Luís, por toda a amizade, incentivo e paciência.

Acima de tudo à minha família... à minha cunhada pela compreensão, à princesinha Diana Sofia, por conseguir fazer-me sempre sorrir, ao meu irmão, pela partilha de experiências e todo o apoio que sempre me deu e aos meus pais, a eles o meu eterno agradecimento, por tudo o que sempre fizeram por mim e por serem os meus pilares não só neste último ano, mas toda a minha vida.

A todos os que me acompanharam nesta jornada pela mui nobre Academia do Minho, o meu muito obrigado, foi uma honra poder partilhar tantos momentos, melhores ou piores, convosco!

Estes anos são viagem!

ABSTRACT

The pharmaceuticals compounds are environmental micropollutants, which are not completely removed in wastewater treatment plants. Contamination by pharmaceutical compounds have increased in the last years and the negative effects in the environment, and in public health, imposes investigating new possible ways of decontamination and treatment. Anaerobic digestion for the treatment of wastewater may be a possibility, coupling degradation of organic matter to biogas production. Though being a slow process, degradation rates can be enhanced by the addition of conductive materials as redox mediators, such as magnetite, activated carbon, carbon nanotubes (CNT) and nanocomposites of carbon and magnetic nanomaterials, as for example, CNT impregnated with iron (CNT@Fe). Notwithstanding, the application of anaerobic digestion and carbon nanomaterials for the treatment of pharmaceutical compounds, requires the knowledge of their impact on the methanogenic communities. In this way, the main goal of this work was to evaluate the individual effect of four pharmaceuticals, Ciprofloxacin (CIP), Diclofenac (DCF), Ibuprofen (IBP) and 17α-ethinylestradiol (EE2), and two nanomaterials, CNT and CNT@2%Fe, towards specific trophic groups from a methanogenic community. Moreover, the effect of CNT and CNT@2%Fe was tested in the anaerobic removal of three pharmaceuticals, DCF, IBP and EE2. CIP removal was not studied, since it was previously assayed in the group.

The results of this work revealed that hydrogenotrophic activity was almost not inhibited by the pharmaceuticals in study (CIP, DCF, IBP or EE2) in all concentrations tested, indicating the low sensibility of the hydrogenotrophic methanogens to these compounds. Overall, the methanogenic communities were most affected by CIP and EE2, followed by DCF and IBP, being the acetoclastic archaea the most sensitive group to the presence of these micropollutants. CNT and CNT@2%Fe did not presented toxicity towards the anaerobic sludge. Furthermore, the methanogenic activity was stimulated in their presence, mainly by CNT (28 ± 4 %).

Neither DCF nor IBP could be biotransformed by the anaerobic sludge, even with CNT or CNT@2%Fe. Nevertheless, approximately 42 % of EE2 could be removed anaerobically and about 60 % were removed in the assays conducted with the nanomaterials tested. However, complete removal of EE2 was achieved in abiotic assays with CNT or CNT@2%Fe. Further research is necessary to understand the mechanisms of EE2 removal.

These materials appear to be good options to be used in the anaerobic treatment of pollutants, both as strong adsorbents and as redox mediators, since they stimulated the microbial methanogenic communities. Despite the biodegradation results are still preliminary, it presented good forecasts for the EE2.

***Keywords*:** Anaerobic biodegradation; Nanomaterials; Pharmaceuticals; Specific methanogenic activity; Toxicity.

RESUMO

Os compostos farmacêuticos são microcontaminantes ambientais que não são completamente eliminados nas estações de tratamento de águas residuais. A contaminação por compostos farmacêuticos tem vindo a aumentar nos últimos anos e terá efeitos negativos no ambiente e na saúde pública, sendo urgente investigar novas formas de descontaminação e tratamento. O tratamento de águas residuais através da digestão anaeróbia poderá ser uma possibilidade, permitindo simultaneamente a degradação de poluentes e a produção de biogás. A digestão anaeróbia é um processo lento, no entanto pode ser melhorado através da adição de materiais condutores como mediadores redox, tais como a magnetite, carvão ativado, nanotubos de carbono, e nanocompósitos de carbono e nanomateriais magnéticos, como por exemplo, CNT e CNT impregnados com de ferro, CNT@Fe. No entanto, a aplicação da digestão anaeróbia ou adsorção por nanomateriais de carbono para o tratamento de fármacos requer conhecimento dos potenciais efeitos destes compostos nas comunidades metanogénicas. Desta forma, o principal objetivo deste trabalho foi avaliar o efeito de quatro fármacos, ciprofloxacina (CIP), diclofenaco (DCF), ibuprofeno e 17α-etinilestradiol (EE2), e de dois nanomateriais, CNT e CNT@2%Fe, em grupos tróficos específicos das comunidades metanogénicas. Para além disso, testou-se o efeito dos CNT e CNT@2%Fe na remoção anaeróbia de três fármacos, DCF, IBP e EE2. A remoção da CIP não foi testada, visto que esse trabalho já foi efetuado em estudos anteriores.

Os resultados revelaram que a atividade hidrogenotrófica praticamente não foi inibida pelos fármacos em estudo (CIP, DCF, IBP, EE2), independentemente da concentração testada, indicando a baixa sensibilidade dos microrganismos hidrogenotróficos a estes compostos. No geral, as comunidades metanogénicas foram mais afetadas pela CIP e pelo EE2, seguidas pelo DCF e pelo IBP, tendo sido o grupo de microrganismos acetoclásticos o mais sensível à presença destes micropoluentes. Para além disto, a atividade metanogénica foi estimulada na presença de CNT e CNT@2%Fe, principalmente pelos CNT
(28 ± 4 %).

Tanto o DCF como o IBP não foram biotransformados pela comunidade anaeróbia, com ou sem CNT ou CNT@2%Fe. No entanto, obteve-se aproximadamente 42 % de remoção de EE2 na ausência de nanomateriais de carbono e cerca de 60% na sua presença. É ainda de notar que se observou a remoção completa de EE2 nos ensaios abióticos com CNT ou CNT@2%Fe. Contudo, novos estudos são necessários para se compreender os mecanismos de remoção da EE2.

Estes materiais aparentam ser boas opções na utilização em tratamentos anaeróbios de poluentes, como adsorventes, mas também como mediadores redox, visto que estimularam as comunidades metanogénicas. Apesar de os resultados de degradação serem preliminares, apresentam boas perspetivas para aplicações futuras no tratamento de EE2.

Palavras-chave: Atividade metanogénica específica; Biodegradação anaeróbica; Fármacos; Nanomateriais; Toxicidade.

TABLE OF CONTENTS

Agradecimentos iii
Abstract v
Resumo vii
Table of contents ix
List of Figures xiii
List of Tables xv
List of Abbreviations xix
1. State of the art 1
1.1 Pharmaceutical waste in the environment and wastewater treatment 3
1.1.1 Processes for removal of pharmaceuticals from wastewaters 4
1.1.2 Characteristics of the pharmaceuticals used in this work 6
1.1.3 Economic advantages of pharmaceuticals treatment through anaerobic digestion 7
1.1.4 Effect of pharmaceuticals in methanogenic communities 8
1.2 Anaerobic Digestion 9
1.2.1 Hydrolysis 10
1.2.2 Acidogenesis 11
1.2.3 Acetogenesis 11
1.2.4 Methanogenesis 11
1.3 Effect of conductive nanomaterials in electrons transfer 12
1.4 Carbon nanotubes and magnetic nanocomposites: characteristics and applications 14
2. Aims and motivation 19
3. Toxicity of pharmaceuticals towards methanogenic communities 23
3.1 Introduction 25
3.2 Materials and Methods 26
3.2.1 Effect of pharmaceuticals towards methanogenic communities 26
3.2.1.1 Calculation of the inhibitory percentage 28
3.2.2 Statistical Analysis 28
3.3.1 Effect of ciprofloxacin on SMA 29

3.3.2 Effect of diclofenac on SMA 30
3.3.3 Effect of ibuprofen on SMA 32
3.3.4 Effect of 17α-ethinylestradiol on SMA 32
3.4 Discussion 34
4. Effect of CNT and CNT@2%Fe on the activity of methanogenic communities 37
4.1 Introduction 39
4.2 Materials and Methods 40
4.2.1 Experimental setup 40
4.2.2 Characteristics of the nanomaterials used 40
4.2.3 Statistical Analysis 40
4.3 Results 41
4.4 Discussion 41
5. Biodegradation assays of pharmaceuticals 43
5.1 Introduction 45
5.2 Materials and Methods 46
5.2.1 Experimental setup for the removal of pharmaceuticals 46
5.2.1.1 Analysis of pharmaceuticals by UHPLC 47
5.2.1.2 Analysis of methane by GC 48
5.2.1.3 Analysis of VFA by HPLC 49
5.3 Results 49
5.3.1 Removal of diclofenac, ibuprofen and 17α-ethinylestradiol 49
5.3.2 Consumption of VFA and CH_4 production in the removal assays of pharmaceutical compounds 50
5.4 Discussion 53
6. Conclusions and future perspectives 59
References 63
7. Appendices 73
Appendix I - Specific methanogenic activity tests 75
Calculation of methanogenic acetoclastic and acetogenic activity 75

Calculation of methanogenic hydrogenotrophic activity 76
Appendix II - Preparation of stock solutions 76
Appendix III - Statistical analysis of the toxicity of pharmaceuticals towards methanogenic communities 78
Appendix IV – Effect of ibuprofen from stock solutions prepared with acetonitrile, methanol or ethanol, and of the solvents towards methanogenic communities 85
Appendix V – Methanogenic activity in ethanol 87
Appendix VI - UHPLC and HPLC calibration curves 88
Appendix VII - Chromatograms of biodegradation 89

LIST OF FIGURES

Figure 1.1 – Dissemination route of pharmaceuticals. Source: Stalder *et al.,* 2012. 3

Figure 1.2 - Anaerobic digestion of wastes with efficient outlet for organic waste stream and generation of valuable products. Source: Lettinga *et al.,* 2001. 8

Figure 1.3 - Reactive scheme for the anaerobic digestion of polymeric materials. 1 – Hydrolytic and fermentative bacteria, 2 – Acetogenic bacteria, 3 – Homo-acetogenic bacteria, 4 – Hydrogenotrophic methanogens, 5 – Acetoclastic methanogens. Source: Lier *et al.*, 2008. 10

Figure 1.4- Types of interspecies electron exchange identified in methanogenic environments. A - indirect interspecies electron transfer via soluble/diffusable chemical compounds (hydrogen); B - Direct Interspecies Electron Transfer; C - Electron Exchange Via Insoluble Electron Shuttles; D - Electron Exchange Via Conductive Material. Source: Holmes & Smith, 2016. 13

Figure 1.5 - Graphene and carbon nanotubes as (A) single wall carbon nanotube (SWCNT) and (B) multi-wall carbon nanotube (MWCNT) structures. Different types of SWNTs functionalization: (C) defect-site functionalization, (D) covalent sidewall functionalization, non-covalent functionalization through, (E) surfactants or (F) rolling with polymers. Adapted from: Hirsch, 2002 and Vidu *et al.*, 2014. 14

Figure 7.1 – UHPLC Chromatograms (A) and calibration curve (B), of DCF at the concentrations 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 mg/L. 88

Figure 7.2 – UHPLC Chromatograms (A) and calibration curve (B), of IBP in the concentrations 0.625, 1.25, 2.4, 5, 10 and 20 mg/L. 88

Figure 7.3– UHPLC Chromatograms (A) and calibration curve (B), of EE2 in the concentrations 20, 10, 5, 2.5, 1.25, 0.625 and 0.313 88

Figure 7.4 – HPLC calibration curve of (A) - Acetate; (B) -Butyrate ; (C) - Propionate and (D) – Ethanol in the concentrations of 30, 60, 120, 315, 630, 1250 and 2500 mg/L 89

Figure 7.5 - UHPLC chromatograms at 230 nm of the diclofenac removal assays with CNT and VFA: (A) Biological assay at 0 h reaction; (B) Biological assay at 48 h reaction; (C) Abiotic control at 0 h of reaction and (D) Abiotic control at 48 h of reaction. 89

Figure 7.6 - UHPLC chromatograms at 230 nm of the ibuprofen removal assays with CNT and VFA: (A) Biological assay at 0 h reaction; (B) Biological assay at 48 h reaction; (C) Abiotic control at 0 h of reaction and (D) Abiotic control at 48 h of reaction. 90

Figure 7.7 - UHPLC chromatograms at 225 nm of the 17α-ethinylestradiol removal assays with VFA: (A) - Biological assay at 0 h reaction; (B) - Biological assay at 48 h reaction; (C) - Abiotic control at 0 h of reaction; (D) - Abiotic control at 48 h of reaction; and 17α-ethinylestradiol removal assays with CNT and VFA: (E) - Biological assay at 0 h reaction; (F) - Biological assay at 48 h reaction; (G) - Abiotic control at 0 h of reaction and (H) - Abiotic control at 48 h of reaction 90

LIST OF TABLES

Table 1.1 - Unit processes and operations used for pharmaceutical removal and respective performances. Adapted from: Jung *et al.,* 2015. 5

Table 1.2 – Main characteristics and structure of the pharmaceuticals ciprofloxacin, 17α-ethinylestradiol, ibuprofen and diclofenac. Source: Sigma-Aldrich. 6

Table 3.1 – Experimental conditions for the determination of the toxicity effect of pharmaceuticals towards methanogenic communities. 27

Table 3.2 – Specific methanogenic activity ($mLCH_4$@SPT/gVSS.day) and percentage of SMA inhibition (I), in the presence of different substrates, at increasing concentrations of ciprofloxacin, when CIP was added either as aqueous solution or as powder. 29

Table 3.3 - Specific methanogenic activity ($mLCH_4$@SPT/gVSS.day) and percentage of SMA inhibition (I) in the presence of different substrates at increasing concentrations of diclofenac, when DCF was added either as aqueous solution or as powder. 31

Table 3.4 - Specific methanogenic activity ($mLCH_4$@SPT/gVSS.day) and percentage of SMA inhibition (I) in the presence of different substrates at 100 mg/L of ibuprofen (IBP was added as powder) and without IBP..... 32

Table 3.5 - Specific methanogenic activity ($mLCH_4$@SPT/gVSS.day) and percentage of SMA inhibition (I) in the presence of different substrates at increasing concentrations of 17α-Ethinylestradiol, when EE2 was added either from a stock solution containing ethanol or as powder (100 mg/L). The SMA and inhibition of control for the effect of organic solvent (OSS) is also presented. 33

Table 4.1 - Methanogenic activity on VFA and stimulation (%), without and with 100 mg/L CNT or CNT@2%Fe. 41

Table 5.1 – Concentration of pharmaceuticals and organic solvents (used to dissolve IBP and EE2) in the biodegradation assays, and reaction times analysed..... 47

Table 5.2 – Gradient of mobile phase for the UHPLC analysis of IBP. A, 15 mM phosphate buffer, pH 3.25 and B, acetonitrile 48

Table 5.3 - Removal extent (%) of DCF, IBP and EE2 at different conditions after 48 h of reaction. For the conditions not showed the removal was zero..... 50

Table 5.4 – Concentration of VFA at 0 h (t0) and 48 h (t48) of reaction, and methane produced after 48 h of reaction, at the different conditions 51

Table 5.5 – Concentration of VFA at 0 h (t0) and 48 h (t48) of reaction, and methane produced after 48 h of reaction, at the different conditions 51

Table 5.6 – Concentration of VFA and of ethanol at 0 h (t0) and 48 h (t48) of reaction, and methane produced after 48 h of reaction, at the different conditions. 52

Table 5.7 – Percentages of removal (R) reported in literature for removal of diclofenac, and the conditions of the treatment..... 56

Table 5.8 - Percentages of removal (R) reported in literature for removal of ibuprofen, and the conditions of the treatment..... 56

Table 5.9 - Percentages of removal (R) reported in literature for removal of 17α-ethinylestradiol, and the conditions of the treatment 57

Table 7.1 - Concentrations of the different solutions of IBP and EE2 present in the tests vials and corresponding concentrations of organic solvent..... 77

Table 7.2 – Summary of the statistical results comparing the effects of the different concentrations of ciprofloxacin in the methanogenic activity in the presence of different substrates. 78

Table 7.3 - Summary of the statistical results comparing the effects of the different concentrations of diclofenac in the methanogenic activity in the presence of different substrates. 79

Table 7.4 - Summary of the statistical results, comparing the effects of the different concentrations of ibuprofen, dissolved in acetonitrile, and the effect of acetonitrile, in the methanogenic activity, in the presence of different substrates. 80

Table 7.5 - Summary of the statistical results, comparing the effects of the different concentrations of ibuprofen, dissolved in methanol, and the effect of methanol, in the methanogenic activity in the presence of different substrates. 82

Table 7.6 - Summary of the statistical results, comparing the effects of the 100 mg/L of ibuprofen, dissolved in ethanol, and the effect of ethanol, in the methanogenic activity, in the presence of different substrates. 83

Table 7.7 - Summary of the statistical results, comparing the effects of the different concentrations of 17α-ethinylestradiol, dissolved in ethanol, and the effect of ethanol, in the methanogenic activity in the presence of different substrates. 83

Table 7.8 - Specific methanogenic activity ($mLCH_4$@SPT/gVSS.day) and percentage of SMA inhibition (I) in the presence of different substrates, at increasing concentrations of ibuprofen, prepared from stock solutions containing acetonitrile, methanol or ethanol. The SMA and inhibition of control, for the effect of organic solvent with substrate (OSS) and without substrate (OS), is also presented 86

Table 7.9 - Specific methanogenic activity ($mLCH_4$@SPT/gVSS.day) in the presence of different concentrations of ethanol and without substrate (OS). 87

LIST OF ABBREVIATIONS

AC – Activated carbon

ACN – Acetonitrile

AD – Anaerobic digestion

AnWT – Anaerobic Wastewater Treatment

AOP – Advances oxidation processes

AcS – Activated sludge

AS – Anaerobic sludge

B – Blank

BAC – Biological activated carbon;

CIP – Ciprofloxacin

CM – Carbon nanomaterials

CNT – Carbon nanotubes

CNT@2%Fe - Composites of carbon nanotubes impregnated with 2% of iron

DIET – Direct Interspecies Electron Transfer

DCF – Diclofenac

EE2 – 17α-ethinylestradiol

GAC – Granular activated carbon

HPLC – High Performance Liquid Chromatography

IBP – Ibuprofen

IC_{50} – Concentration that induces an inhibition of 50%

IET – Interspecies electron transfer

IIET – Indirect interspecies electron transfer

LCFA – Long chain fatty acids

MMNC – Metal matrix nanocomposites

MWCNT – Multiwalled carbon nanotubes

NF – Nanofiltration;

NSAID - Non-steroidal anti-inflammatory drug

OM – Organic matter

OS – Organic solvent without substrate

OSS – Organic solvent with substrate

pH_{pzc} - pH of zero charge

pK_a – Ionization constant

RO – Reverse osmosis;

Rpm – rotations per minute

RM – Redox mediator

SC – Substrate control

SMA – Specific methanogenic activity

SRT – Sludge retention time

STP – Standard pressure and temperature

SWCNT – Single walled carbon nanotubes

UV – Ultraviolet;

UHPLC – Ultra-High Performance Liquid Chromatography

VFA – Volatile fatty acids

VS – Volatile solids

WWTP – Wastewater treatment plants

1. STATE OF THE ART

1.1 Pharmaceutical waste in the environment and wastewater treatment

Nowadays, the most pressing global issues we are facing is the concomitant environmental degradation (Jorgenson, 2003). This was aggravated with the industrial revolution, which led to a rapidly grow of the global energy demands and production of synthetic organic chemicals, with consequent increase in waste generation (Panayotou, 1993; Stern *et al.*, 1996; Donohoe, 2003). With advances in modern pharmacology, since the late 19th century, consumption of pharmaceuticals by humans, as well as their utilization in veterinary practices had a drastic increase (Cetecioglu *et al.,* 2013; Rodrigues, 2013; Sousa, 2015). These implies higher loads of pharmaceuticals into wastewater treatment plants (WWTP) through excretion of the consumed pharmaceuticals as well as through the effluents from hospitals private household, industries or farms (Figure 1.1) (Stalder *et al.,* 2012).

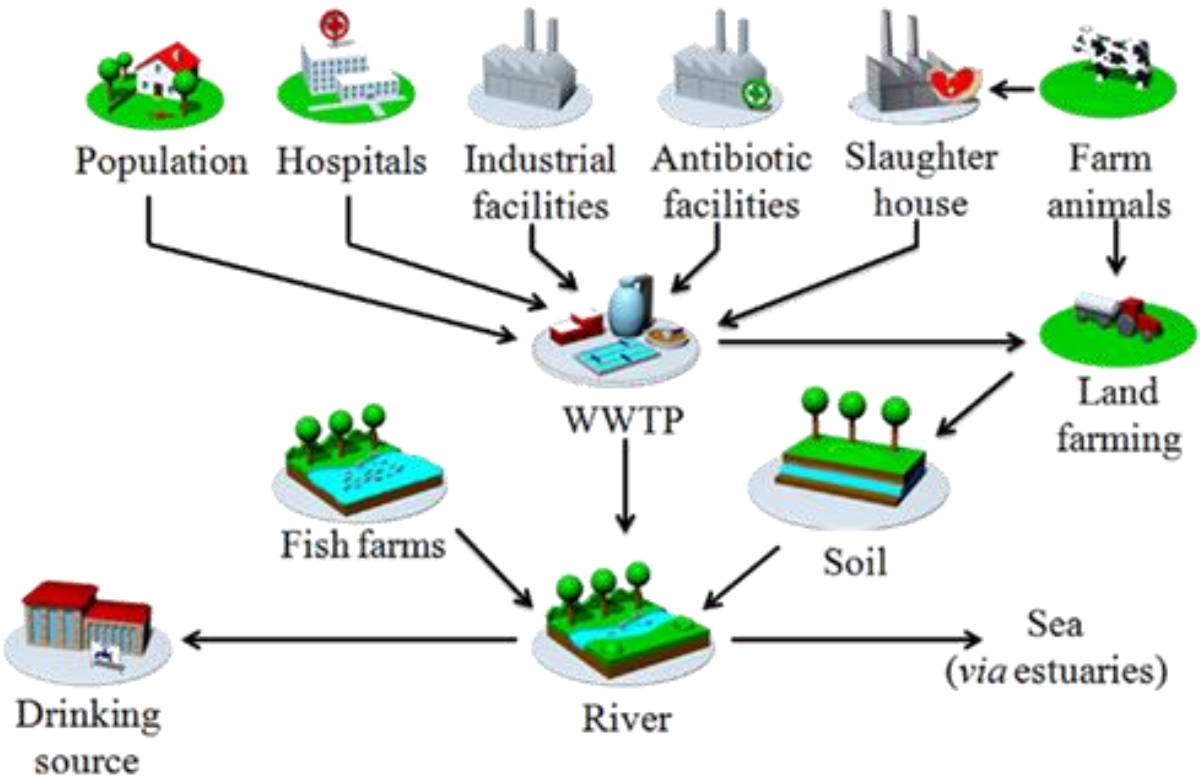


Figure 1.1 – Dissemination route of pharmaceuticals. Source: Stalder *et al.,* 2012.

Some of these pharmaceuticals, such as non-steroid anti-inflammatory drugs, as diclofenac (DCF) and ibuprofen (IBP); antibiotics, like ciprofloxacin (CIP); antidepressants and anxiolytics, as for example lorazepam; lipid regulators, as simvastatin, and the synthetic estrogenic hormone 17α-ethinylestradiol (EE2), have been detected in concentrations ranging from 125 ng/L to 66 µg/L in Portuguese wastewater treatment plants (WWTP) (A. Pereira *et al.,* 2015; Sousa, 2015). Despite the

concentration of pharmaceuticals in water varying in concentrations in the range of nanograms to micrograms per litre, these pharmaceuticals when concentrate in sludge reach concentrations of 58 $mg_{pharmaceutical}/Kg_{sludge}$ (U.S. EPA, 2009). These compounds are considered emergent micropollutants, due to their physical and chemical properties, pharmacokinetic characteristics (such as absorption, distribution, metabolism and excretion), biotransformation resistance, environmental persistence and accumulation in organisms, entering this way, in the food chain (Nunes, 2010; Sousa, 2015; Campbell, 2017). In addition, when these micropollutants pass in a WWTP they are not completely mineralized, staying partially retained in the sludge, or being metabolized to a more hydrophilic form, that disseminate into the surface water, groundwater and even drinking water (Figure 1) (Nunes, 2010; Carvalho *et al.*, 2013; Cetecioglu *et al.,* 2013).

1.1.1 Processes for removal of pharmaceuticals from wastewaters

Complete degradation of pharmaceuticals in conventional WWTP is difficult, therefore, with the increase conscience about the possible effects of these residues in the environment and in public health, various approaches for their treatment have been proposed to remove them, preventing their dissemination. This approaches include: sedimentation, hydrolysation, chlorination, advanced oxidation processes (AOP), ozonation, photolysis, sorption or biodegradation (Table 1.1) (Nunes, 2010; Cetecioglu *et al.*, 2013; Jung *et al.*, 2015). For example, 91 % of degradation of DCF was achieved by Hartmann and collaborators (2008) applying ultrasonic irradiation. These methodologies have different efficiencies, being the removal through AOP and ozonation the best when compared to the remaining methods (Table 1.1). Despite the promising results achieved with these technologies, they present high costs, high energy consumption, and can possibly produce residual toxic by-products (Jung *et al.*, 2015; Hasan *et al.*, 2016; Campbell, 2017). Interesting results have been achieved with adsorption-based techniques (Table 1.1), which represent a simple operation system with minimal energy consumption (Jung *et al.*, 2015; Hasan *et al.*, 2016). For example, addition of activated carbon resulted in 99 % removal rate of antidepressants and anxiolytics (Sousa, 2015).

Over the past few years, some studies focused on biodegradation of pharmaceuticals, achieving good performances. For example complete degradation of 1.7 μM of DCF via co-metabolism with acetate, was achieved over a period of 6 days (Moreira *et al.* 2018) . Moreover, when DCF (1.7–34 μM) was used as the sole carbon source, 70 % biotransformation of the pharmaceutical was obtained by the activity of the bacterial strain *Labrys portucalensis* F11, in 30 days (Moreira *et al.,* 2018).

Table 1.1 - Unit processes and operations used for pharmaceutical removal and respective performances. Adapted from: Jung *et al.*, 2015

Classification	AC	BAC	CNT	O_3/ AOPs	UV	Cl_2/ ClO_2	Coagulation/ flocculation	Softening/ metal oxides	NF	RO	Degradation {B/P/AcS}
Steroids	E	E	G-E	E	E	E	P	P-L	G	E	L-E {B}
Antibiotics	F-G	E	F-E	L-E	F-G	P-G	P-L	P-L	E	E	E {B} GE {P}
Antidepressants	G-E	G-E	F-E	E	F-G	P-F	P-L	P-L	G-E	E	G-E
Anti-inflammatories	E	G-E	F-E	E	E	P-F	P	P-L	G-E	E	E {B}
Lipid regulators.	E	E	G-E	E	F-G	P-F	P	P-L	G-E	E	P {B}

AC – Activated carbon; CNT – Carbon nanotube; BAC – Biological activated carbon; AOPs – Advanced oxidation processes; UV – Ultraviolet; NF – Nanofiltration; RO – Reverse osmosis; {B} – Biodegradation; {P} – Photodegradation (solar); {AcS} – Activated sludge; E – Excellent (>90%); G - Good (70-90%); F – Fair (40-70%); L – Low (20-40%) and P – Poor (<20%).

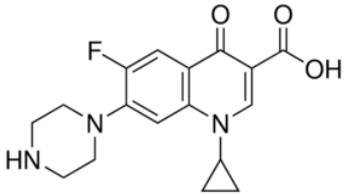
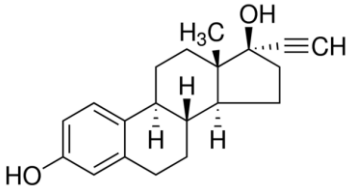
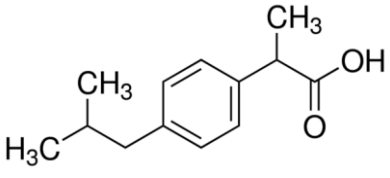
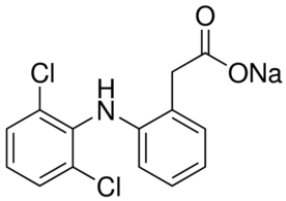
Biodegradation studies under anaerobic conditions have been conducted as well, and interesting results were achieved (Carballa *et al.*, 2007; Musson *et al.* 2010; Gonzalez-Gil *et al.* 2018). However, degradation rates vary greatly depending on the pharmaceutical, due to differences in their chemical structure and ionization constant (pK_a), and on the systems conditions, as the presence of oxygen or an appropriate microbial community, acclimation time, pH and temperature (Jung *et al.*, 2015; Campbell, 2017). For example, increasing temperature and supplementing glucose or ammonia nitrogen can increase CIP biodegradation by microbial communities, where members belonging to *Gammaproteobacteria*, *Bacteroidia*, and *Betaproteobacteria* were present (Liao *et al.,* 2016). Musson and collaborators (2010) reported an acetylsalicylic acid reduction of 16 % after 56 days of anaerobic digestion (AD). In a lab-scale anaerobic baffled reactor, 15–68 % of removal were achieved for tetracycline antibiotic (Lu *et al.*, 2016). Additionally, Butkovskyi and collaborators (2015) were able to remove oxazepam, ibuprofen, metoprolol and diclofenac in an up-flow anaerobic sludge (AS) blanket at percentages of 71.9 %, 32.5 %, 72.0 % and 12.3 %, respectively. Moreover, Cetecioglu and collaborators (2013) reported 70 % of sulfamethoxasol biodegradation in 20 days under methanogenic conditions and in 60 days under nitrate and sulphate reducing conditions. Lower percentages were achieved by Santos and collaborators in 2014, with 18 % removal of norfloxacin in anaerobic digesters.

1.1.2 Characteristics of the pharmaceuticals used in this work

Some pharmaceuticals have been found to have mutagenic and carcinogenic effects, to cause endocrine disruption, even at low concentrations, and increase bacterial resistance by antibiotics, having impacts at various levels: single cells, organs, organisms, populations and ecosystems (Rodrigues, 2013). Additionally, in the sludge, they can create an unbalance between the different microbial communities, impacting the AD (M. S. Fountoulakis *et al.*, 2008; Ji *et al.*, 2013; Hom-Diaz *et al.*, 2016). In this way, it becomes necessary to know the effects of the pharmaceuticals in the environment, and to find effective forms of treatment.

CIP (Table 1.2) is a member of fluoroquinolones, which acts by binding to the DNA gyrase complex, disrupting bacterial DNA replication and repair and, consequently, leading to bacteriostasis and cell death (Ji *et al.*, 2013; Mai *et al.*, 2018). Up to 72 % of CIP is excreted as the parent compound, which accumulation in the environment, leads to problems related to genotoxicity and also increases the antibiotic resistance of pathogenic bacteria. This compound has high ecological risk even at the levels currently detected in the environment (Liu *et al.*, 2013; Mai *et al.*, 2018).

Table 1.2 – Main characteristics and structure of the pharmaceuticals ciprofloxacin, 17α-ethinylestradiol, ibuprofen and diclofenac. Source: Sigma-Aldrich.

Ciprofloxacin	**17α-ethinylestradiol**
	
Molecular weight: 331.34 g/mol pK_a: 6.09 Antibiotic	Molecular weight: 296.4 g/mol pK_a: 10.5 Synthetic hormone
Ibuprofen	**Diclofenac**
	
Molecular weight: 206.3 g/mol pK_a: 4.9 Anti-inflammatory	Molecular weight: 318.13 g/mol pK_a: 4.15 Anti-inflammatory

DCF (Table 1.2) is a recalcitrant non-steroidal anti-inflammatory drug (NSAID) detected worldwide in environmental samples (including drinking water), with removal efficiency in WWTP typically lower than

40 % (Sari *et al.*, 2013; Cherik *et al.*, 2015; Symsaris *et al.*, 2015). It has potential toxic effects on non-target organisms, such as birds, fishes, crustaceans and algae, and damage microbial cell walls at concentrations of 5 μg/L (Fountoulakis *et al.*, 2008; Cherik *et al.*, 2015; Hu *et al.*, 2018).

Up to 15% of IBP (Table 1.2), is non-metabolized, being excreted in its initial form, so being problematic when reaching aquatic systems, presenting toxicity towards aquatic organisms, such as fish and algae, which may alter the food chain and, consequently, the ecosystem (Quero-Pastor *et al.*, 2014; Campbell, 2017).

EE2 is a synthetic oestrogen that mimic or block the activity of endogenous hormones (Table 1.2) (Ribeiro *et al.*, 2010). This pharmaceutical is an endocrine-disrupting chemical, which causes negative effects on the endocrine systems of fish and mammals, even at levels of ng/L. Moreover, the increase concentration of EE2 and prevalence of cancer in the endocrine system of humans have been correlated (Ribeiro *et al.*, 2010).

1.1.3 Economic advantages of pharmaceuticals treatment through anaerobic digestion

With an increasing concern about our environmental management strategies and the need to reduce energy consumption, developing sustainable clean technologies to waste treatment and assuring environmental sustainable bioenergy production became a priority (Stern *et al.*, 1996; Lettinga *et al.*, 2001; Yang *et al.*, 2010). Renewable energy, has emerged as an alternative to fossil fuels, aiming, among other benefits, to effectively reduce CO_2 emissions and minimize related global warming and climate change impacts (Weiland, 2010; Lijó *et al.*, 2017). Biogas offers significant advantages over other forms of bioenergy production. It has been evaluated as one of the most energy-efficient and environmentally beneficial technology for bioenergy production (Weiland, 2010). It is produced by AD of organic matter and is primarily composed of methane and carbon dioxide, but also contains small amounts of hydrogen sulphide and ammonia, and is saturated with water vapor (Weiland, 2010; Senghor *et al.,* 2016). Modern biogas-based systems have major objectives within societies, which are supplying renewable energy and utilising organic wastes from various sources. The process can be applied at a small and large scales, with low costs and achieves sustainable management of waste streams, moving away from current linear economy to the circular one (Weiland, 2010; Lijó *et al.*, 2017). Thus, conversion of waste to biogas will play a vital role in the future, since sewage sludge and industrial effluents become an important source of valuable products, such as water, fertilisers, soil conditioners and energy, after AD, instead of a social threat, as can be seen in Figure 1.2 (Lettinga *et al.*, 2001; Lovley, 2008; Weiland, 2010).

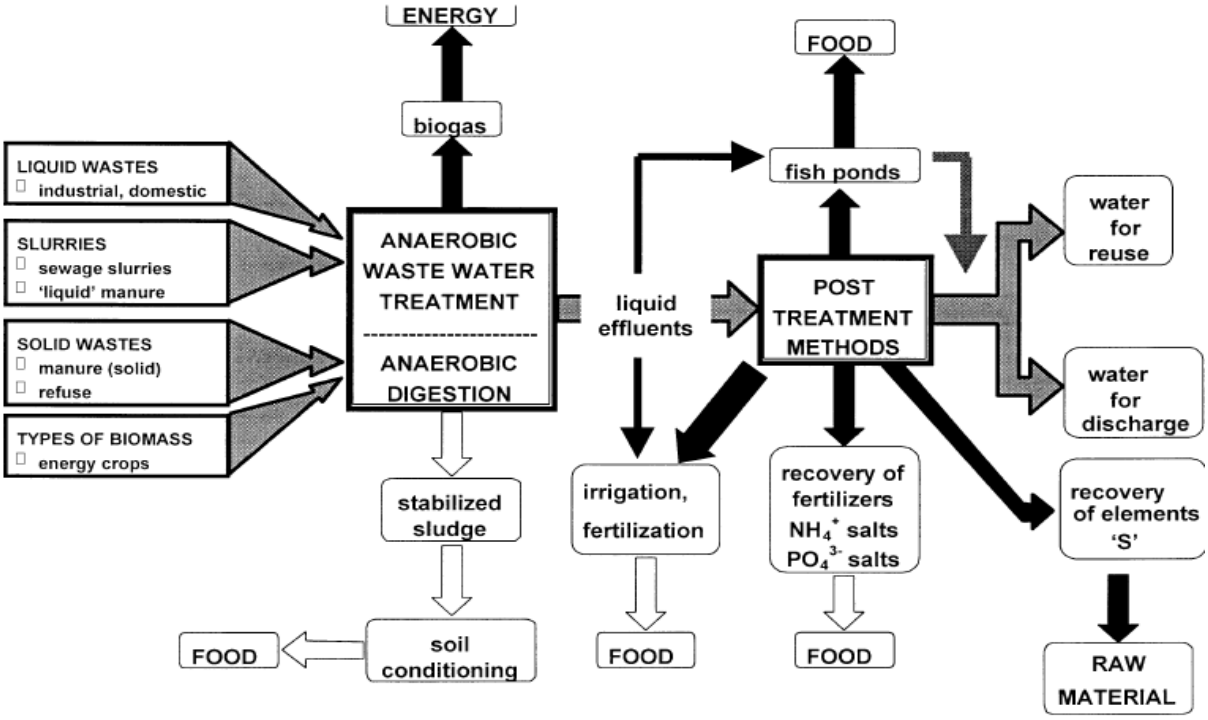


Figure 1.2 - Anaerobic digestion of wastes with efficient outlet for organic waste stream and generation of valuable products. Source: Lettinga *et al.*, 2001.

1.1.4 Effect of pharmaceuticals in methanogenic communities

The application of AD for the treatment of pharmaceutical compounds requires the knowledge of their impact on the methanogenic communities, in order to guarantee the microbial activity of the sludge. Some studies analysing the effect of models of pharmaceuticals as CIP, DCF, IBP and EE2 towards methanogenic communities are available. For example, Liu *et al.* (2013), Mai *et al.* (2018) and Zhao *et al.* (2018) demonstrated CIP inhibitory effect at concentrations of 10, 0.5 and 0.53 mg/L, respectively. An IC_{50} of 4.8 mg/L for CIP was calculated in the study of Mai *et al.* (2018). In the case of DCF, the reported inhibitory concentrations towards methanogenic microorganisms were higher: IC_{50} of 120 mg/L when AS was fed with acetate, casein and yeast extract (Fountoulakis *et al.*, 2004) and 546 mg/L with acetate (Symsaris *et al.*, 2015). However, it should be noticed, that when assessing the inhibition towards specific trophic groups, Hu *et al.* (2018) showed that DCF at concentrations equal or higher than 0.2 mg/L DCF presented toxicity towards acetoclastic communities. Relatively to the effect of IBP and EE2 towards the methanogenic communities, studies conducted by Campbell (2013), Campbell (2017) and Hom-Diaz *et al.* (2016) did not detect any toxic effect of IBP until concentrations of 206 mg/L for IBP and 2 mg/L for EE2.

1.2 Anaerobic Digestion

Anaerobic digestion consists of a series of biological processes, involving a complex food web, in which complex organic wastes are sequentially converted by a wide variety of microorganisms of several different species (Ahn *et al.*, 2010; Lier *et al.*, 2008; Senghor *et al.,* 2016). These are converted into stable molecules containing a single carbon and other compounds forming biogas, by a microbial consortium (Ahn *et al.*, 2010; Lier *et al.*, 2008; Senghor *et al.,* 2016). Despite of these molecules being responsible for greenhouse effect, the controlled conversion of organic waste, like sewage, to methane has been widely recognized as one of the most effective and sustainable technology for simultaneous waste treatment and biogas generation, used as an alternative source of renewable energy, without adverse environmental effect (Ahn *et al.,* 2010; Ziganshin *et al.*, 2013; Liu *et al.,* 2017).

Compared to conventional Aerobic Wastewater Treatment systems, the Anaerobic Wastewater Treatment (AnWT) offers some advantages, such as: applicability at any scale; low space, costs and nutrient requirement; small volume of excess sludge, generally well stabilised and with high dewatering capacity; no or very little use of chemicals; high loading potential and removal efficiencies for organic pollutants; production of energy and transformation of effluents into valuable products (Lettinga *et al.*, 2001; Lier *et al.*, 2008; Weiland, 2010). Although the various advantages of AnWT, there are some drawbacks that are being circumvent with knowledge development of the microbial processes (Sträuber *et al.*, 2016) and acceptance of post-treatment requirement like: detoxification of biotransformed xenobiotic compounds, applying a secondary aerobiotic treatment; process stabilization; diminishing the start-up time, by availability of large quantities of highly active AS from existing full scale installations, and prevention of mal-odorous nuisance problems (Lier *et al.*, 2008; Amezquita-Garcia *et al.,* 2016; Pereira *et al.,* 2016).

The AD is a multistep process of series and parallel reactions, that proceeds in four successive stages, namely: hydrolysis, acidogenesis, acetogenesis - catalysed by distinct bacteria - and methanogenesis - carried out by specialized groups of methanogenic archaea (Figure 1.3) (Lier *et al.*, 2008).

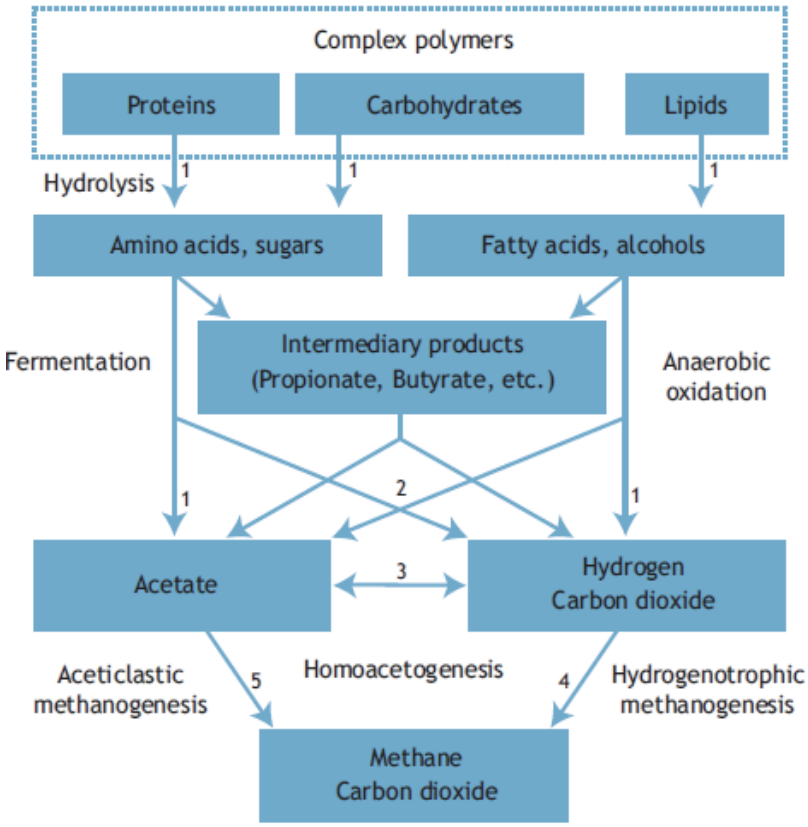


Figure 1.3 - Reactive scheme for the anaerobic digestion of polymeric materials. 1 – Hydrolytic and fermentative bacteria, 2 – Acetogenic bacteria, 3 – Homo-acetogenic bacteria, 4 – Hydrogenotrophic methanogens, 5 – Acetoclastic methanogens. Source: Lier *et al.,* 2008.

1.2.1 Hydrolysis

The hydrolysis convert complex, undissolved organic structures into less complex, dissolved compounds which can pass through the cell walls and membranes, being readily accessible for the acidogenic bacteria (Lier *et al.*, 2008; Yang *et al.*, 2010). Lipids are hydrolysed to long chain fatty acids (LCFA), proteins to aminoacids and polysaccharide to simple sugars (Lier *et al.*, 2008). This degradation of biodegradable particulate organic matter heavily depends on the hydrolytic enzymes secreted by hydrolytic bacteria (so called "exo-enzymes"), like glucosidases, lipases, and proteases (Lier *et al.*, 2008; Yang *et al.*, 2010). The hydrolysis process was investigated for the last three decades and demonstrated to improve sludge digestibility and reducing digesting time and disposal costs, to transform compounds from a recalcitrant state to one that is more biodegradable and to enhance the degree of dewaterability of anaerobically digested biosolids. Moreover this process is very sensitive to temperature fluctuations, making the design of anaerobic digester based on this step (Park *et al.*, 2005; Lier *et al.*, 2008; Yang *et al.*, 2010).

1.2.2 Acidogenesis

During the acidogenesis step, the products of hydrolysis are diffused inside the bacterial cells and subsequently fermented or anaerobically oxidized into simple compounds which are then excreted. These compounds are mainly volatile fatty acids (VFA) and some alcohols, lactic acid, CO_2, H_2, NH_3 and H_2S, as well as new cell material (Lier *et al.*, 2008; Sträuber *et al.*, 2016). These reactions can only be performed in anoxic conditions, since these compounds can be oxidized in the presence of alternative electron acceptors, like oxygen (McInerney *et al.*, 2008). Hydrogen produced in this phase needs to be consumed by methanogenic microorganisms since the acetogenesis phase depends on low hydrogen partial pressure due to thermodynamic constraints (Adekunle & Okolie, 2015).

The intermediate products that are formed in this step, resulting from the metabolic activity of acidogenic bacteria and from syntrophic acetogenic bacteria and methanogenic archaea in the subsequent phases, depend on the conditions in the reactor medium (Lier *et al.*, 2008; Sträuber *et al.*, 2016). The free energy change of acidifying reactions is the most negative of all AD conversions ($\Delta G^0<0$), so it is the faster conversion step in the AD, resulting in higher bacterial growth rates, higher bacterial yields and conversion rates (Lier *et al.*, 2008; Sträuber *et al.*, 2016).

1.2.3 Acetogenesis

The short chain fatty acids, like propionate and butyrate, and alcohols, which are produced in the acidogenesis step, are (homo)acetogenically converted into acetate, H_2 and CO_2, as well as new cell material, by acetogenic bacteria (Stams, 1994; Lier *et al.*, 2008). These latter anaerobic oxidation reactions are highly endergonic ($\Delta G^0>>0$), due to the obligatory production of hydrogen by these bacteria, which inhibits their metabolism and therefore it is possible only when there is a narrow syntrophic association with H_2-consuming methanogenic archaea (Stams, 1994; Lier *et al.*, 2008; Liu *et al.*, 2017).

1.2.4 Methanogenesis

Methanogenic archaea are microorganisms that utilize a limited number of substrates. They convert H_2/CO_2, formate, acetate and a few other substrates like methanol, ethanol, isopropanol, methylamines, methylated sulphur compounds, and pyruvate into methane (a gas highly insoluble in water), CO_2 and new cell material (Stams, 1994; Lier *et al.*, 2008). In methanogenic environments, only fermentation or respiration processes with protons or bicarbonate as electron acceptors are possible, since organic compounds are degraded in the absence of inorganic electron acceptors. Due to the

restricted metabolism of methanogens, associations of fermenting, acetogenic and methanogenic microorganisms, are obligatory (Stams, 1994; Lier *et al.*, 2008). Depending on the electron donor, methanogenesis proceeds through three different pathways:

(i) Hydrogenotrophic methanogenesis: CO_2 is reduced with hydrogen as the electron donor. This reaction is the most favourable, thermodynamically, Equation 1.1 (Zinder, 1993);

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \qquad (\Delta G'^0 = -131\ kJ/mol)$$ Equation 1.1

(ii) Methylotrophic methanogenesis: Methyl groups act as the electron donors in a reaction performed by methylotrophic organisms as *Methanosphaera stadtmanii,* Equation 1.2 (Zinder, 1993);

$$CH_3OH + H_2 \rightarrow CH_4 + H_2O \qquad (\Delta G'^0 = -113\ kJ/mol)$$ Equation 1.2

(iii) Acetoclastic methanogenesis: the electron donor is acetate. This reaction is the least exergonic, as exemplified in Equation 1.3, being performed by *Methanosarcina* and *Methanotrix* organisms (Zinder, 1993).

$$CH_3{}^-COO^- + H_2O \rightarrow CH_4 + HCO_3{}^- \qquad (\Delta G'^0 = -31\ kJ/mol)$$ Equation 1.3

Although these pathways differ in the electron donor, the last steps of the reduction to methane are identical, and catalysed by the Methyl-coenzyme M reductase, a core enzyme present in all known methanogens. The gene *mcrA*, which encodes the α-subunit of this enzyme, is widely used as a marker for the detection of methanogens in the environment (Meslé *et al.*, 2013).

1.3 Effect of conductive nanomaterials in electrons transfer

According to Schink (1997), the classical definition of syntrophy is explained as: "*Cooperation in which both partners depend on each other to perform the metabolic activity observed and in which the mutual dependence cannot be overcome by simply adding a co-substrate or any type of nutrient*". Syntrophic activity produces a set of chemical outcomes that are different from what could occur by each microbe separately (Morris *et al.*, 2013). Therefore, can be considered as a special case of symbiosis between metabolically different types of microorganisms which depend on each other for degradation of a certain substrate. This cooperation enables endergonic reactions to become exergonic through the efficient removal of products, allowing a microbial community to survive with minimal energy resources (Schink, 1997; Morris *et al.*, 2013). In fact, AD relies on the cooperation of numerous microorganisms along a metabolic cascade. While, methanogens need the fermenting microbes to produce their substantial metabolic products, the activity of the methanogenic archaea permits that the other partner

metabolises the substrate by electron exchange (Thiele & Zeikus, 1988; Morita *et al.*, 2011; Meslé *et al.,* 2013). Disruptions in the syntrophic associations between bacteria and methanogens can result in system instabilities during anaerobic digestion (Liu *et al.,* 2017). Therefore, effective interspecies electron transfer (IET) is critical in AD processes and understanding these mechanisms is the key for modelling and/or manipulating methane production (Morita *et al.*, 2011; Liu *et al.,* 2017). Three different types of IET have been identified in methanogenic environments: Indirect Interspecies Electron Transfer (IIET) Via Soluble/Diffusable Chemical Compounds; Direct Interspecies Electron Transfer (DIET) and Electron Exchange Via Insoluble Electron Shuttles and Conductive Materials (Figure 1.4) (Holmes & Smith, 2016).

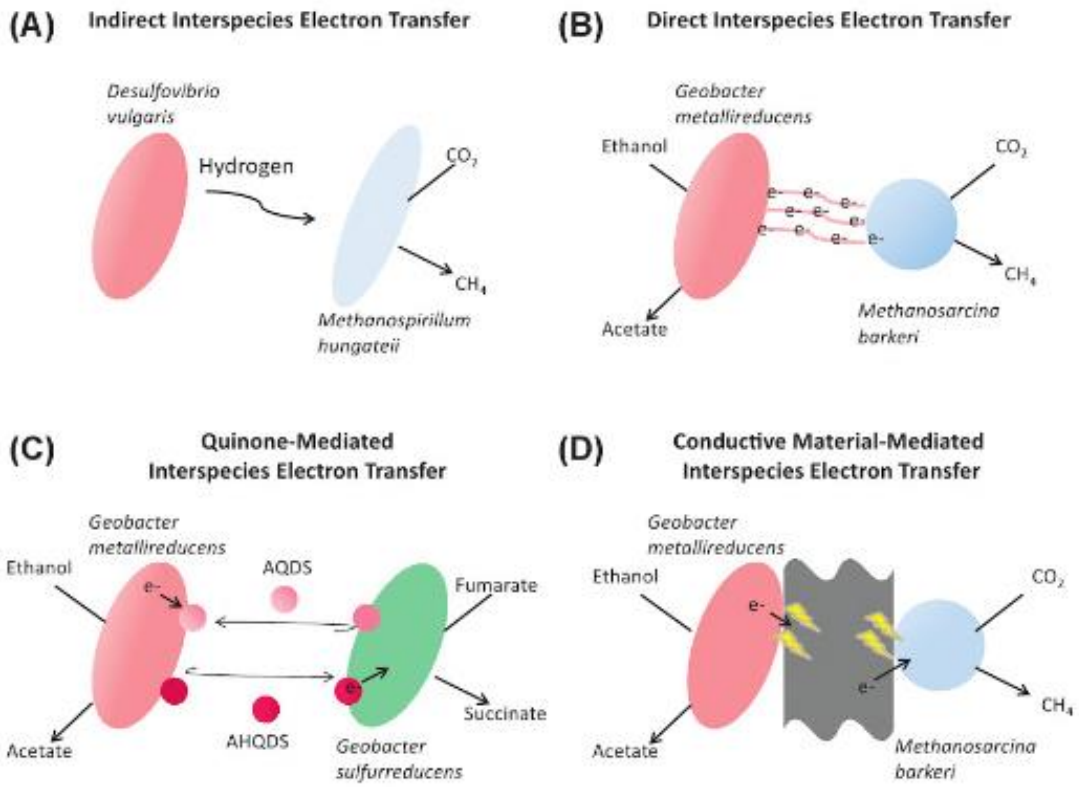


Figure 1.4- Types of interspecies electron exchange identified in methanogenic environments. A - indirect interspecies electron transfer via soluble/diffusable chemical compounds (hydrogen); B - Direct Interspecies Electron Transfer; C - Electron Exchange Via Insoluble Electron Shuttles; D - Electron Exchange Via Conductive Material. Source: Holmes & Smith, 2016.

The IIET via soluble/diffusable chemical compounds is the process where the IET occurs between different microorganisms through compounds as H_2 or formate, which act as electron shuttles (Morita *et al.*, 2011). An alternative IET via soluble/diffusible chemical compounds occurring in methanogenic environments is DIET (Rotaru *et al.*, 2014b; Holmes & Smith, 2016). In this mechanism, species exchange electrons via direct electrical connections, rather than producing electron carriers as in the previous process (Summers *et al.*, 2010; Lovley, 2011; Rotaru *et al.*, 2014b). Another type of IET involves

insoluble electron carriers, like humic substances, sulphur compounds and flavins, and conductive materials. These electron shuttles are large and insoluble, preventing its entrance in cells, therefore facilitating the transfer of electrons outside the cell, similarly to DIET (Shrestha & Rotaru, 2014; Holmes & Smith, 2016).

1.4 Carbon nanotubes and magnetic nanocomposites: characteristics and applications

CNT consists of seamless graphene cylinders with a diameter rounding a few nanometres and several micrometres in length. These structures include multiwalled (MWCNT) and single walled carbon nanotubes (SWCNT) (Camargo *et al.,* 2009; Ong *et al.,* 2010). While SWCNT are made of a single rolled graphite sheet held together by van der Waals interactions and MWCNT are composed for a series of concentric cylindrical graphene sheets coaxially arranged around a central hollow core with spacing between the layers (Figure 1.5) (Chen *et al.,* 2009; Ong *et al.,* 2010; Zhang *et al.,* 2013; Vidu *et al.,* 2014). Besides the number of walls difference, these varieties differ as well in their specific surface area, which is higher in SWCNT, and elastic modulus and strength, lower in SWCNT (Xie *et al.,* 2005).

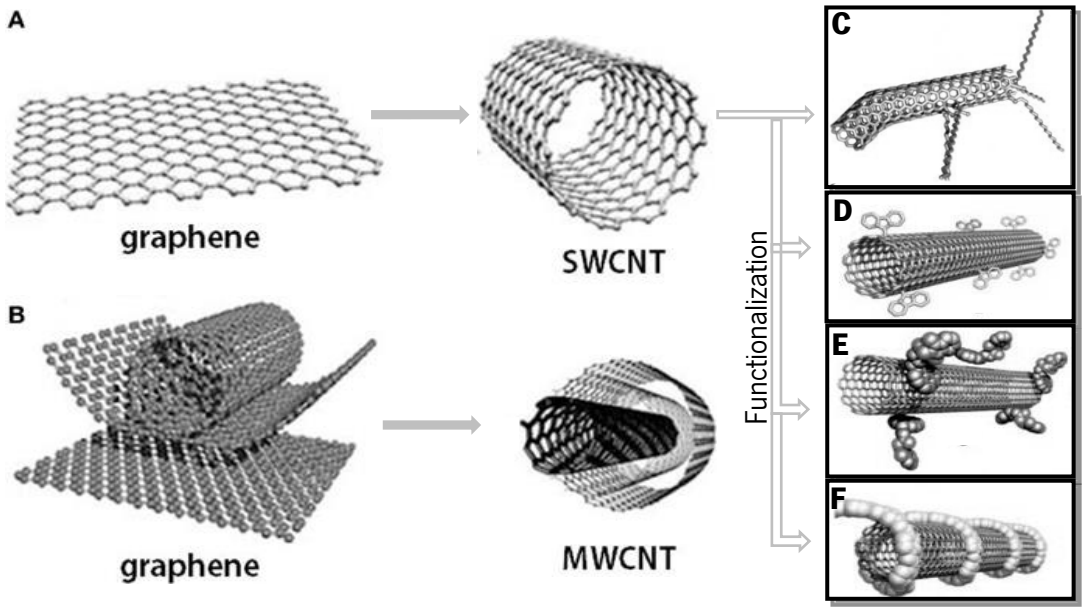


Figure 1.5 - Graphene and carbon nanotubes as (A) single wall carbon nanotube (SWCNT) and (B) multi-wall carbon nanotube (MWCNT) structures. Different types of SWNTs functionalization: (C) defect-site functionalization, (D) covalent sidewall functionalization, non-covalent functionalization through, (E) surfactants or (F) rolling with polymers. Adapted from: Hirsch, 2002 and Vidu *et al.*, 2014.

Due to their conductive nature, large specific surface area, light mass density, high tensile strength and highly porous and hollow structure that characterize CNT, they can be used in diverse applications as energy storage and conversion devices; sensors and supports for catalyst and adsorption materials (Hirsch, 2002; Xie *et al.*, 2005; L. Li *et al.*, 2015). In addition, these properties make them a feasible catalyst and redox mediator (RM) (R. Pereira *et al.*, 2014), facilitating DIET, as reported by L. Pereira and collaborators (2016) and Zhang & Lu (2016). This is supported by a study from Li and collaborators (2015) that demonstrated that SWCNT induce substrate consume and double methane production rates. However, using the same CNT concentration and temperatures, Yan *et al.* (2017) did not observe noticeable effects on the methanogenic activity.

Ambuchi and collaborators (2017) and Salvador and colleagues (2017), demonstrated an increase in methanogenic activity using CM. In the first study, iron oxide nanoparticles and MWCNT, promoted methane production 28.9 % and 12.6 %, respectively, and in the study of Salvador *et al.* (2017) MWCNTs accelerated the methane production by pure cultures of acetoclastic and hydrogenotrophic methanogens. Moreover, Tian and collaborators (2013) investigated the adsorption characteristics of sulfamethoxazole and sulfapyridine on CNT and verified that these antibiotics had very fast adsorption kinetics to the materials, similarly with the results documented by Ji and collaborators with MWCNTs (2009). Additionally, investigators studied the adsorption efficiency of other pharmaceuticals on these materials (Pan *et al.,* 2008, 2010; Joseph *et al.*, 2011a, 2011b), achieving a maximum removal percentages of 95 - 98 % for EE2. Cho *et al.*, (2011) obtained 40 % of adsorption of IBP on SWCNT and MWCNT and Sotelo *et al.* (2012) determined the adsorption capacity for DCF, 41.4 $mg_{pharmaceutical}/g_{MWCNT}$. Due to the high efficiency of CNT as adsorbents, their use to remove pharmaceuticals from aqueous solution seems very pertinent (Jung *et al.*, 2015). However, CNT may exhibit strong antimicrobial activity towards bacteria, as well as towards bacterial spores (Ong *et al.*, 2010; Sharma *et al.,* 2015). In a study performed by Kang and collaborators (2007) severe membrane damage and subsequent cell inactivation of *Escherichia coli* was observed, when in direct contact with SWCNTs, confirming its strong antimicrobial activities. This toxicity can be related with damage of microorganism major constituents (e.g., cell wall), interference with cellular metabolism and growth inhibition by blockage of the synthesis of key constituents (Ong *et al.*, 2010; Tian *et al.,* 2017). According to Sayes and collaborators (2006), this cytotoxicity activity is dependent on the functionalization of the compounds, decreasing with the augmented degree of sidewall functionalization Therefore, CNT can be modified to a less harmful form for microorganisms (Hirsch, 2002; Ong *et al.*, 2010). Furthermore, a study performed by Li *et al.* (2015) showed that excretion of

extracellular polymeric substances by AS is increased in the presence of SWCNT, diminishing the nanoparticle cytotoxicity.

CNT utilization as sorbents, nanofilters and antimicrobial agents is thus encouraged, due to the favourable mechanical, electrical, physical and chemical properties. Moreover, they improve the sludge settleability and dewaterability, playing a major role in wastewater treatment and methane production efficiency (Ong *et al.*, 2010; Salvador *et al.,* 2017; Tian *et al.,* 2017). However, CNT still present some drawbacks that should be taken into account as their high cost, difficult recovery and reuse and possible environmental impact (Pan *et al.*, 2010; Jung *et al.*, 2015; Martins *et al.*, 2018). This can be lessened by using low amounts of more efficient materials, or by combination with magnetic nanoparticles to turn them more efficient as adsorbers and catalysts, but special allowing their recover easily by a magnetic field, and further reuse.

Nanocomposites are combinations of compounds in which at least one of them is nanosized (range 1 nm). The size, shape and dispersity of nanocomposites is crucial to their properties (Camargo *et al.,* 2009; Pereira *et al.,* 2015). According to nanocomposite matrix of the materials, they can be classified in three different categories: ceramic matrix nanocomposites; metal matrix nanocomposites (MMNC) and polymer matrix nanocomposites. MMNC consist in a material composed by a ductile metal or alloy matrix implanted with some nanosized reinforcement material. Some of these nanoparticles include gold, silver, platinum and iron magnetic oxides, such as magnetite (Fe_3O_4), hematite (α-Fe_2O_3), and nanoscaled zero-valent iron (nFe^0) (Camargo *et al.,* 2009; L. Pereira *et al.,* 2015; L. Pereira *et al.,* 2017).

Metallic nanoparticles exhibit unique properties, like high surface area, magnetic separation, sorption and catalytic characteristics, that make them valuable in different areas, including environmental biotechnological applications (Oliveira *et al.,* 2002; L. Pereira *et al.,* 2015; L. Pereira *et al.,* 2017). However, the small adsorption capacity constitutes a drawback of these materials (Chen *et al.,* 2009). On the other hand, carbon materials are good adsorbents, but their recovery and reuse are difficult and expensive, as stated before for CNT. Therefore, creating magnetic carbon composites by combination of carbon materials and magnetic particles with synergistic properties, opens promising possibilities in the field of adsorption technology. In other words, it is possible to prepare a material with improved adsorptive and catalytic properties and magnetic character, allowing magnetic separation, a low cost, simple, quick and efficient way of separation (L. Pereira *et al.,* 2015; Sharma *et al.,* 2016; L. Pereira *et al.,* 2017). A study on environmental applicability of these new compounds was shown by Oliveira *et al.* (2002). In this study, a magnetic adsorbent was produced by combination of AC and iron oxides, maintaining the surface area and porosity of AC, so that there was no reduction of the adsorption capacity of the AC, but with the

simple recuperation of the material associated to the magnetic characteristics. In addition, the composite showed the capability to adsorb a wide range of contaminants in water (Oliveira *et al.*, 2002). However, the unique properties of CNTs suggests more appealing opportunities for new composites (Xie *et al.*, 2005). Therefore, Chen and collaborators (2009) and L. Pereira and collaborators (2017) conducted experiences with MWCNT impregnated with iron oxide and composites of CNT impregnated with 2 % of iron (CNT@2%Fe), respectively for Ni(II) adsorption and azo dye chemical and biological reduction. In the first study, Ni(II) adsorption and desorption by the composite was verified, showing pH and ionic strength dependency. In the work reported by L. Pereira et al. (2017), it was observed an improvement of the efficiency on the treatment of the azo dye AO10 solution, in the presence of low CNT@2%Fe concentrations, between 0.1 and 0.5 g/L. It is noteworthy that this MMNC was applied in consecutive cycles of AO10 decolourisation efficaciously and its presence increased the rate of the reaction 79 times, when compared with the control. Although there is still need more improvements in nanotechnology systems and research, this suggests a future increasing preference for these materials in water treatment technologies due to their high efficiency and low cost recover when compared to other RM (L. Pereira *et al.,* 2015; L. Pereira *et al.,* 2017).

2. AIMS AND MOTIVATION

Large amounts of pharmaceuticals are used throughout the world, daily, and enter in WWTP where some are recalcitrant and other are not completely mineralized. This way, pharmaceuticals can accumulate becoming a problem of environmental and public health concern, special if they reach ecosystems and enter in the food chain. Therefore, it is important to evaluate new treatment processes. Anaerobic digestion of sewage sludge and wastewater contaminated with pharmaceuticals may be an option to biodegrade these compounds and to generate bioenergy, as methane. Anaerobic digestion treatment is a slow process but may be accelerated in the presence of conductive materials, such as CNT. Notwithstanding, the application of anaerobic digestion and carbon nanomaterials for the treatment of pharmaceutical compounds requires the knowledge of their impact on the methanogenic communities. Therefore, the specific aims of the present project are the following:

1. Evaluate the effect of increasing concentrations of the pharmaceuticals, CIP, IBP, DCF and EE2, on the specific methanogenic activity;
2. Evaluate the effect of CNT and CNT@2%Fe on the methanogenic activity;
3. Follow the biotransformation of selected pharmaceuticals (IBP, DCF and EE2) by an anaerobic sludge, with and without carbon nanomaterials (CNT and CNT@2%Fe).

3. TOXICITY OF PHARMACEUTICALS TOWARDS METHANOGENIC COMMUNITIES

3.1 Introduction

The application of anaerobic digestion for the treatment of pharmaceutical compounds requires the knowledge of their impact on the methanogenic communities. The effect of model pharmaceuticals, such as CIP, DCF, IBP and EE2, towards methanogenic communities was investigated before. Liu *et al.* (2013) reported significant inhibitions only at CIP concentrations of 80 mg/L and 100 mg/L, 30 % and 46 %, respectively, using dextrin and peptone as carbon and energy source. Recent studies carried out by Zhao *et al.* (2018), with anaerobic sludge, demonstrated that at 0.3 mg/L of CIP, in the presence of nutrient broth, yeast extract and glucose as substrate, the methane production rate was delayed by 1 hour and less methane was produced (approximately less 43 %). Using AS, supplemented with glucose, peptone and meat extract, Mai *et al.* (2018) indicated that at concentrations higher than 0.5 mg/L, CIP caused a significant decrease of methane production rate. The IC_{50} predicted through the fitting of experimental values to the three-parameter Gompertz equation was 4.8 mg/L, but the results showed that methanogenic activity still occurred at 50 mg/L, even though methane production rate was reduced in 61 % when compared to the control assay (Mai *et al.,* 2018). In the study of Fountoulakis *et al.* (2004), with AS fed with acetate, casein and yeast extract, DCF caused moderate methanogenesis inhibition at 100 mg/L, and severe inhibition with concentrations higher than 200 mg/L, resulting in an IC_{50} of 120 mg/L. However, Symsaris *et al.* (2015) demonstrated a correlation between the increasing concentrations of DCF and the reduction of methane production yield in AS with acetate, revealing higher values of IC_{50} (546 mg/L) and complete inhibition of methanogenesis at 3000 mg/L of DCF. On the other hand, Hu *et al.* (2018), using waste activated sludge, showed that DCF until 0.7 mg/L stimulated acidogenesis, acetogenesis and homoacetogenesis processes without effects in hydrogenotrophic activity. Nevertheless, at concentrations higher than 0.2 mg/L DCF presented toxicity towards acetoclastic communities, since with the increasing of DCF concentration from 0.04 mg/L to 0.7 mg/L, the methane production decreased from 53 to 29 mL. Relatively to the effect of IBP, a study conducted by Campbell (2013) did not detect any toxic effect towards AS with a concentration of 66 mg/L using glucose, nutrient broth and yeast extract as substrate. More recently, Campbell (2017) using acetate and propionate as substrates revealed that, although IBP caused an initial inhibitory effect of the total gas production, the amended methanogenic culture could recover after a period of 5 days. Besides, the overall total gas production was greater than active controls at 103 and 206 mg/L of IBP tested, revealing that IBP was not toxic to the methanogenic community (Campbell, 2017). Studies assessing the effect of these pharmaceuticals on SMA using only acetate or H_2/CO_2 as the direct substrates of methanogenesis are rare. As it was showed above, the existing literature focus mainly in the use of substrates as complex

polymers or sugars to stimulate all the anaerobic community, instead of evaluating the effect towards specific trophic groups participating in AD. Thus, it is important to evaluate the effect of pharmaceuticals towards key groups of microorganisms involved in AD.

In this chapter, the individual effect of CIP, DCF, IBP and EE2 on the SMA of anaerobic sludge incubated with different carbon and energy sources will be studied. The substrates used (i.e., acetate, hydrogen with carbon dioxide, and a mixture of volatile fatty acids) will be tested in independent assays to target trophic groups of microorganisms with different metabolic specificities, namely acetoclastic methanogens and hydrogenotrophic methanogens and acetogenic bacteria. The pharmaceuticals (CIP, an antibiotic; DCF and IBP, anti-inflammatories, and EE2, an hormone) were chosen considering their occurrence in the environment and in WWTP systems (Langenhoff *et al.*, 2013; A. Pereira *et al.*, 2015).

3.2 Materials and Methods

3.2.1 Effect of pharmaceuticals towards methanogenic communities

The biological material used as inoculum in the toxicity tests was an anaerobic granular sludge collected from the anaerobic digester from Super Bock Group brewery, Porto, Portugal, on September 2017.

The effect of the pharmaceuticals was assessed by determining the SMA in the presence of increasing concentrations of pharmaceuticals (ranging from 0.05 to 100 mg/L corresponding to 17 $mg_{pharmaceutical}/kg_{sludge}$ to 3 $g_{pharmaceutical}/kg_{sludge}$). SMA of the anaerobic sludge was determined as described by Colleran *et al.* (1992) and Coates *et al.* (1996) and is detailed in Appendix I. The SMA was determined in the presence of different substrates that served as carbon and energy source: SMA in H_2/CO_2 (to access the effect of pharmaceuticals on the activity of hydrogenotrophic methanogens); SMA in acetate (to access the effect of pharmaceuticals on the activity of acetoclastic methanogens) and SMA in a mixture of VFA (as a way of indirectly estimate the effect towards acetogenic bacteria). The substrates were provided in the following concentrations: acetate (30 mM), H_2/CO_2 (80:20 % v/v, at 1.7 x 10^5 Pa), VFA mixture (10 mM acetate, 10 mM propionate and 5 mM butyrate). The assays were conducted in bottles of 25 mL of capacity, for liquid substrates, and of 70 mL, for gaseous substrates, both with a working volume of 12.5 mL and a final concentration of anaerobic biomass of 3 gVS/L. The medium used was made up with demineralised water containing resazurin (1 g/L) and sodium bicarbonate (3 g/L). No reducing agent was added and the pH was corrected to 7.0-7.2. After the biomass addition, the bottles were sealed with a butyl rubber and an aluminium capsule, the headspace was flushed with N_2/CO_2 (80:20 % v/v),

depressurized and then incubated overnight (37°C and 110 rpm), for depletion of residual substrate and temperature acclimation. After overnight incubation, the bottles headspace was flushed again with a mixture of N_2/CO_2 (80:20 % v/v), depressurized, and then the substrates were added: 0.125 mL for liquid substrates and H_2/CO_2 (80:20 % v/v) (1 bar overpressure) for gaseous substrates. The blank assay prepared for the gaseous substrate contained N_2/CO_2 (80:20 % v/v) (1 bar overpressure), instead of H_2/CO_2. Blank assays were prepared without pharmaceuticals and without substrate to assess the residual methane produced by the anaerobic communities, and control assays were performed without pharmaceuticals but with substrate, to determine the SMA without pharmaceuticals. All the assays were performed in triplicate.

The anaerobic sludge was incubated with increasing concentrations of CIP, IBP, DCF and EE2 as specified in Table 3.1. The first assays were made with CIP and IBP in concentrations between 0.05 and 10 mg/L. However, these concentrations had low inhibitory effect in the SMA, and so the following assays were made with a higher range of concentrations, until 100 mg/L (Table 3.1). IBP and EE2 have very low solubility in water (21 mg/L at 25 °C and 11.3 mg/L at 27 °C, respectively), but are very soluble in most organic solvents like acetonitrile (ACN), methanol and ethanol (NPCS, 2013), which implied the addition of solvents to promote the dissolution of the pharmaceuticals when preparing the solutions. The preparation of the pharmaceuticals stock solutions is described in the Appendix II.

Table 3.1 – Experimental conditions for the determination of the toxicity effect of pharmaceuticals towards methanogenic communities.

Pharmaceutical	Pharmaceutical (mg/L)	Solvent	Controls
CIP	0.05; 0.5; 1; 5; 10; 50 and 100	Water with HCl[a]	B; SC
DCF	1; 5; 10; 50 and 100	Water	B; SC
IBP	0.05; 0.5; 1; 5; 10 and 20	ACN	B; SC; OSS[b]
	5; 10; 50 and 100	Methanol	B; SC; OSS and OS
	100	Ethanol	B; SC; OSS and OS
EE2	1; 5; 10; 50 and 100	Ethanol	B; SC; OSS and OS

a –250 µL of HCl (2M) were added; b - for concentration of 0.05; 1 and 10 mg/L IBP

Acetonitrile (ACN); Blank (B) – Without substrate, pharmaceutical or solvent; Substrate control (SC)– Only substrate added to the buffer (no pharmaceutical and no solvent); Organic solvent controls– No pharmaceutical addition to the buffer, only the organic solvent in the concentrations correspondent to that of the tests with pharmaceuticals, either with substrate (OSS) or without substrate (OS).

For the pharmaceuticals which were dissolved by addition of organic solvents, it was necessary to perform additional controls (in duplicate). One of the controls was performed in the presence of organic solvent and substrate (OSS), without the pharmaceutical compound, to allow the differentiation between the effect of pharmaceutical and the effect of the organic solvent. A control with the organic solvent, but without substrate (OS), was necessary when the solvents used were methanol or ethanol, since these solvents may also be substrates for the anaerobic communities. Additionally, and in order to access the effect of the pharmaceuticals only, without the interference of the solvents, an extra assay with the addition of 100 mg/L of the pharmaceuticals in powder directly to the bottles was performed.

3.2.1.1 Calculation of the inhibitory percentage

The percentages of inhibition were calculated considering 100% of activity for the control without pharmaceutical, according to Equation 3.1.

Equation 3.1

$$I\ (\%) = \frac{SMA_P}{SMA_{SC}} \times 100$$

Where:

I = Inhibition

SMA_{SC} = Specific methanogenic activity in the substrate control

SMA_P = Specific methanogenic activity in the presence of pharmaceutical

3.2.2 Statistical Analysis

Statistical analysis was performed using the GraphPad software. For comparisons between the effects in the SMA of the different concentrations of the model pharmaceuticals, the Brown-Forsythe test was used. It allowed verifying the homogeneity of variances and, then, the One-Way ANOVA parametric test with a p-value of 0.05 to verify if there were significant differences between the inhibitions associated to the different concentrations of pharmaceuticals and controls. To identify which concentrations were significantly different, the Tukey post-test was applied with significance level of 5 %. The F test was used to compare variances between the assays with pharmaceuticals added as powder versus the substrate control and the pharmaceutical added as solution. The parametric unpaired t test with a p-value of 0.05 allowed the verification of significant differences between the inhibitions associated to the addition of pharmaceutical, as powder or as solution. In the case of different variances, as it succeeded comparing

the acetogenic activity between the EE2, added as powder and solution, the non-parametric unpaired t test, with Welch's correction and a p-value of 0.05, was performed, aiming to verify if there were significant differences between them.

3.3 Results

The anaerobic sludge was active in all the substrates tested as showed by the SMA obtained for the control assays (without addition of pharmaceuticals). The highest SMA was obtained in H_2/CO_2 (515 ± 52 $mLCH_4$@SPT/gVSS.day), followed by acetate (79 ± 11 $mLCH_4$@SPT/gVSS.day) and VFA (82 ± 13 $mLCH_4$@SPT/gVSS.day) (Table 3.2 to Table 3.5).

3.3.1 Effect of ciprofloxacin on SMA

The effect of CIP on the SMA, at the different conditions studied, and the inhibition percentages are presented in Table 3.2.

Table 3.2 – Specific methanogenic activity ($mLCH_4$@SPT/gVSS.day) and percentage of SMA inhibition (I), in the presence of different substrates, at increasing concentrations of ciprofloxacin, when CIP was added either as aqueous solution or as powder.

		Substrate					
		Acetate		**H_2/CO_2**		**VFA mixture**	
Assay	**CIP (mg/L)**	SMA ($mLCH_4$@SPT/gVSS.day)	I (%)	SMA ($mLCH_4$@SPT/gVSS.day)	I (%)	SMA ($mLCH_4$@SPT/gVSS.day)	I (%)
Solution	0	91 ± 7*	0	599 ± 11*	0	89 ± 2*	0
	0.05	99 ± 3	0	583 ± 21	0	65 ± 5	10 ± 9
	0.5	94 ± 9	0	616 ± 20	0	60 ± 7	18 ± 10
	1	76 ± 3	21 ± 4	606 ± 15	0	53 ± 6	27 ± 9
	5	77 ± 6	15 ± 10	598 ± 8	0	66 ± 5	27 ± 5
	10	86 ± 4	0	589 ± 21	0	64 ± 9	29 ± 9
	50	52 ± 6	40 ± 6	592 ± 9	0	77 ± 5	28 ± 5
	100	51 ± 5	42 ± 5	572 ± 9	4 ± 2	72 ± 1	32 ± 2
Powder	0	76 ± 2	0	502 ± 18	0	66 ± 1	0
	100	19 ± 3	76 ± 4	486 ± 9	0	52 ± 4	21 ± 6

For values with higher standard deviation than the median values, and negative values of the percentage of inhibition, were considered zero.
*Mean from the SMA in substrate control from the two solution assays (SMA of substrate control in the first assay was 95 ± 5 $mLCH_4$@SPT/gVSS.day in acetate, 603 ± 22 $mLCH_4$@SPT/gVSS.day in H_2/CO_2 an 73 ± 4 $mLCH_4$@SPT/gVSS.day in VFA mixture; SMA of substrate control in the second assay was 87 ± 14 in acetate, 596 ± 6 in H_2/CO_2 an 106 ± 3 in VFA mixture).

The results showed that CIP affected the methanogenic activity with acetate and VFA as substrates but the hydrogenotrophic activity was almost not affected (Table 3.2). For example, in acetate, SMA decreased from (91 ± 7) $mLCH_4$@SPT/gVSS.day to approximately 52 $mLCH_4$@SPT/gVSS.day in concentrations of 50 and 100 mg/L of CIP (added as solution), and in VFA from (89 ± 2) $mLCH_4$@SPT/gVSS.day to (72 ± 1) $mLCH_4$@SPT/gVSS.day in concentrations of 100 mg/L of CIP (added as solution) (Table 3.2). Acetoclastic activity was significantly affected by CIP at the concentrations of 50 and 100 mg/L of CIP, while the SMA in VFA decreased significantly already at CIP concentrations higher than 1 mg/L of CIP (Table 3.2 and Table 7.2 in Appendix III). Surprisingly, as can be observed in Table 3.2, 10 mg/L of CIP affected less the methanogenic acetoclastic activity than 1 and 5 mg/L CIP (21 and 15 % of inhibition, respectively), though not being a significant difference (Table 7.2 in Appendix III).

A statistical analysis was performed to determine if the effect of CIP (100 mg/L of powder) was significant or not. The results showed that significant differences between the assays with CIP and the assays without CIP were only observed in the SMA from acetate (p values < 0.0001) and VFA (p value = 0.0042), while no significant differences were obtained for the SMA from H_2/CO_2. The differences between the inhibition caused by CIP (100 mg/L) from a stock solution or powder were evaluated. Significant differences were only observed in the SMA from acetate (p value = 0.0073) with an increase in the inhibition from (40 ± 5) % (CIP in solution) to (76 ± 4) % (CIP in powder) (Table 3.2), while no significant differences were obtained for the SMA with VFA or H_2/CO_2.

Summing up, for concentrations up to 10 mg/L of CIP, methanogenic activity in VFA was the most affected. At concentrations higher than 10 mg/L of CIP methanogenic activity in both acetate and VFA was affected, but the highest inhibition was observed towards the acetoclastic methanogens. The hydrogenotrophic activity was almost not affected in all concentrations tested.

3.3.2 Effect of diclofenac on SMA

The effect of DCF on the SMA at the different conditions studied and inhibition percentages are presented in Table 3.3.

Table 3.3 - Specific methanogenic activity (mLCH_4@SPT/gVSS.day) and percentage of SMA inhibition (I) in the presence of different substrates at increasing concentrations of diclofenac, when DCF was added either as aqueous solution or as powder.

		Substrate					
		Acetate		**H_2/CO_2**		**VFA mixture**	
Assay	**DCF (mg/L)**	SMA (mLCH_4@SPT/gVSS.day)	I (%)	SMA (mLCH_4@SPT/gVSS.day)	I (%)	SMA (mLCH_4@SPT/gVSS.day)	I (%)
Solution	0	62 ± 4	0	498 ± 63	0	99 ± 6	0
	1	74 ± 4	0	507 ± 32	0	96 ± 2	0
	5	59 ± 1	0	532 ± 8	0	93 ± 3	0
	10	66 ± 1	0	505 ± 68	0	87 ± 5	13 ± 8
	50	51 ± 1	18 ± 5	531 ± 10	0	84 ± 4	15 ± 6
	100	38 ± 1	39 ± 4	539 ± 10	0	72 ± 5	28 ± 8
Powder	0	76 ± 2	0	502 ± 18	0	66 ± 1	0
	100	47 ± 6	38 ± 8	530 ± 21	0	68 ± 1	0

For values with higher standard deviation than the median values, and negative values of the percentage of inhibition, were considered zero.

The results obtained with DCF showed a low toxic effect towards methanogenic activity, being the highest inhibition (around 39 %) observed in methanogenic acetoclastic activity for the assays with 100 mg/L of DCF, provided either in solution or in powder (Table 3.3). Nevertheless, inhibition of the SMA from acetate and from VFA increased with increasing DCF concentrations. For example in acetate, the inhibition increased from (18 ± 5) % to (39 ± 4) %, when biomass is exposed to 50 and 100 mg/L of DCF, and in VFA, from (13 ± 8) % to (28 ± 8) %, when biomass is exposed to 10 and 100 mg/L of DCF (Table 3.3); achieving significant differences in both cases at concentrations of 50 and 100 mg/L of DCF relatively to the control without DCF (Table 7.3 in Appendix III - Statistical analysis of the toxicity of pharmaceuticals towards methanogenic communities). This was not observed for the SMA from hydrogen, since differences in SMA from hydrogen, in all conditions tested, were not statistically significant (Table 3.3, Table 7.3 in Appendix III).

When DCF was added as powder (100 mg/L), only the acetoclastic activity was inhibited, with a significant reduction in the SMA of (38 ± 8) % (p value of 0.0014), which was similar to the assay with the addition of DCF at the same concentration but as solution (Table 3.3). However, with VFA, (28 ± 7) % inhibition of SMA was obtained when DCF was added from stock solution and no inhibition was observed when DCF was added as powder (Table 3.3). In VFA, the SMA inhibition obtained between the assays

with 100 mg/L of DCF from stock solution or as powder, was significant (p value = 0.0018), decreasing from (28 ± 8) % to 0 % (Table 3.3).

Summing up, DCF inhibited methanogenic activity in both acetate and VFA for concentrations of 50 and 100 mg/L. The hydrogenotrophic activity was not inhibited in all concentrations tested.

3.3.3 Effect of ibuprofen on SMA

To evaluate the toxicity of IBP towards the methanogenic community, incubations with IBP dissolved in several solvents (ACN, Methanol and Ethanol) were performed. The results obtained are difficult to interpret because the solvents used, either inhibited (i.e., ACN and Methanol), or stimulated the biomass activity (i.e., Ethanol) (Table 7.4, Table 7.5 and Table 7.6 in Appendix III). The effect of the solvents used towards the methanogenic activity is presented in Table 7.8 in Appendix IV. Therefore, only the results obtained with IBP in powder (without any solvent) will be considered for discussion (Table 3.4). The results showed that IBP (100 mg/L) inhibited significantly the acetoclastic activity (20 ± 6) % (p value = 0.0073) and had no effect in the SMA in H_2/CO_2 or VFA (Table 3.4).

Table 3.4 - Specific methanogenic activity ($mLCH_4$@SPT/gVSS.day) and percentage of SMA inhibition (I) in the presence of different substrates at 100 mg/L of ibuprofen (IBP was added as powder) and without IBP.

IBP (mg/L)	**Substrate**					
	Acetate		**H_2/CO_2**		**VFA mixture**	
	SMA ($mLCH_4$@SPT/gVSS.day)	I (%)	SMA ($mLCH_4$@SPT/gVSS.day)	I (%)	SMA ($mLCH_4$@SPT/gVSS.day)	I (%)
0	76 ± 2	0	502 ± 18	0	66 ± 1	0
100	63 ± 4	20 ± 6	515 ± 3	0	70 ± 3	0

For values with higher standard deviation than the median values, and negative values of the percentage of inhibition, were considered zero.

3.3.4 Effect of 17α-ethinylestradiol on SMA

To evaluate the toxicity of EE2 towards methanogenic community, incubations with EE2 dissolved in ethanol were performed and variations in SMA and respective inhibitions were analysed (Table 3.5). Similarly to what happened in the assay with IBP dissolved in ethanol, it served as additional substrate for the methanogenic community (Table 7.9 in Appendix V), which affected the SMA. Nevertheless, for higher concentrations of EE2 (50 mg/L and 100 mg/L), which corresponded to lower concentrations of ethanol (1 M and 0.64 M, respectively), the effect of the solvent was less evident, which allowed to observe a significant inhibitory effect towards the acetoclastic methanogens, associated to the pharmaceutical

(Table 7.7 in Appendix III): approximately 24 % for 50 mg/L of EE2, and 48 % with 100 mg/L of EE2. The same was observed for 50 mg/L of EE2 in VFA, where an inhibition of approximately 21 % associated to the pharmaceutical was achieved.

Table 3.5 - Specific methanogenic activity ($mLCH_4$@SPT/gVSS.day) and percentage of SMA inhibition (I) in the presence of different substrates at increasing concentrations of 17α-Ethinylestradiol, when EE2 was added either from a stock solution containing ethanol or as powder (100 mg/L). The SMA and inhibition of control for the effect of organic solvent (OSS) is also presented.

		Substrate					
Solution assay		**Acetate**		**H_2/CO_2**		**VFA mixture**	
		SMA ($mLCH_4$@SPT/gVSS.day)	I (%)	SMA ($mLCH_4$@SPT/gVSS.day)	I (%)	SMA ($mLCH_4$@SPT/gVSS.day)	I (%)
EE2 (mg/L)	0	87 ± 3	0	459 ± 25	0	75 ± 7	0
	1	58 ± 4	33 ± 5	254 ± 18	45 ± 5	50 ± 1	33 ± 6
	5	60 ± 6	31 ± 7	299 ± 10	35 ± 4	54 ± 6	28 ± 11
	10	51 ± 2	41 ± 3	329 ± 12	28 ± 5	49 ± 2	34 ± 7
	50	60 ± 4	31 ± 5	306 ± 5	33 ± 4	61 ± 1	19 ± 7
	100	68 ± 3	21 ± 5	273 ± 6	41 ± 3	87 ± 6	0
Ethanol[a] (M)	1.36	61 ± 3	30 ± 5	284 ± 7	38 ± 4	43 ± 2	42 ± 6
	1.33	57 ± 3	34 ± 4	255 ± 48	44 ± 11	44 ± 1	41 ± 5
	1.30	56 ± 1	35 ± 3	301 ± 13	34 ± 5	63 ± 2	16 ± 8
	1.00	81 ± 1	7 ± 4	315 ± 17	31 ± 5	76 ± 1	0
	0.64	110 ± 4	0	266 ± 34	42 ± 8	89 ± 2	0
EE2 (mg/L) powder	0	76 ± 2	0.	502 ± 18	0	66 ± 1	0
	100	37 ± 2	51 ± 3	523 ± 9	0	51 ± 1	23 ± 2

a – in the presence of substrate.

For values with higher standard deviation than the median values, and negative values of the percentage of inhibition, were considered zero. The ethanol concentrations of 1.36, 1.33, 1.30, 1 and 0.65 M correspond to the ethanol concentrations present in the assays performed with 1, 5, 10, 50 and 100 mg/L EE2, respectively.

When EE2 was added as powder, the hydrogenotrophic activity was not affected but the SMA from acetate and VFA decreased (from (76 ± 2) $mLCH_4$@SPT/gVSS.day to (37 ± 2) $mLCH_4$@SPT/gVSS.day in acetate and from (66 ± 1) $mLCH_4$@SPT/gVSS.day to (51 ± 1) $mLCH_4$@SPT/gVSS.day in VFA) with significant differences when compared to the control, p value < 0.0001 (Table 3.5).

All EE2 concentrations from the stock solution containing ethanol inhibited significantly the methanogenic hydrogenotrophic activity, however, the inhibition is similar to the corresponding OSS, and therefore does not reflect the effect of EE2 (Table 7.7 in Appendix III).

For effects of comparison between the inhibition of EE2 when added as powder or solution to the medium, the effect of EE2 as solution was considered as the difference between test vials of 100 mg/L of EE2 and the respective OSS. In this way, inhibition associated to the presence of 100 mg/L of EE2 added from stock solution was 48 % in acetate and 21 % in VFA mixture. Inhibition between the pharmaceutical added from stock solution and as powder was similar for all substrates, with no significant differences detected.

Summing up, comparing the SMA in the presence of EE2 with the correspondent OSS, it is possible to conclude that EE2 inhibited methanogenic activity in both acetate and VFA for concentrations of 50 and 100 mg/L. The hydrogenotrophic activity was not inhibited in all concentrations tested.

3.4 Discussion

CIP, DCF, IBP and EE2 affected the methanogenic activity, at least in one of the substrates used. Overall, the methanogenic communities were most by CIP and EE2, followed by DCF and IBP. Although the percentage of inhibition in the assays of EE2 with H_2/CO_2 were high (around 36 %, Table 3.5), these were close to the inhibitions obtained in the respective OSS (around 38 %, Table 3.5). This way, the methanogenic acetoclastic activity was the most affected, with higher SMA inhibition percentages when compared to the inhibition of the SMA in VFA and H_2/CO_2. In fact, the acetoclastic activity was affected by all pharmaceuticals tested. This can be related with the higher sensitivity of the acetoclastic archaea to the presence of these micropollutants, followed by the acetogenic bacteria. The hydrogenotrophic methanogens were almost not affected. The results are in accordance with the expected, since literature reported that the acetoclastic methanogens are more sensitive to environmental changes, while hydrogenotrophic methanogens present higher robustness (Schon, 2009).

For concentrations up to 10 mg/L of CIP, methanogenic activity in VFA was the most affected (Table 3.2). At concentrations higher than 10 mg/L of CIP, the methanogenic activity in both acetate or VFA was affected, but with higher inhibition of the acetoclastic activity, indicating that acetogenic bacteria is more sensitivity to the presence of CIP. However, at higher concentrations of the antibiotic, the most affected group are the acetoclastic methanogens. This toxicity may be associated to the fact that CIP is an antibiotic that acts by binding to the DNA gyrase complex, leading to cell death (Ji *et al.*, 2013; Mai *et*

al., 2018). In this way, it was expected to observe toxic effects, mainly in the SMA with VFA. Besides inhibition of the bacteria groups, inhibition of the archaea was also expected, since in the order *Methanosarcinales* (which includes the genera of acetoclastic methanogens, *Methanosarcina* and *Methanosaeta*), DNA gyrase is also found, as for example in *Methanosarcina barkeri* (Gadelle *et al.*, 2003). The toxic effect of DCF is related to its action as inhibitor of the DNA synthesis, acting on specific bacteria and on certain anaerobic archaea (Fountoulakis *et al.*, 2008), which could explain the inhibition of the SMA with acetate and VFA in the higher concentrations tested (50 and 100 mg/L) (Table 3.3). Moreover, DCF inhibits the activity of F_{420}, a coenzyme involved in redox reactions in methanogens, both acetoclastic and hydrogenotrophic (Hu *et al.*, 2018). However, in this work no inhibitory effect was observed in the SMA from H_2/CO_2. The inhibitory effect of IBP was only verified in the methanogenic acetoclastic activity, indicating that the acetoclastic archaea are more sensitive to the presence of IBP than acetogenic bacteria or hydrogenotrophic archaea. IBP is a NSAID, with reported anti-microbial properties (Campbell, 2017). A toxicity study with EE2 at 2 mg/L did not presented any toxic effect towards methanogenic communities (Hom-Diaz *et al.* 2016), which is in agreement with the results obtained in this work for the lower concentration tested. EE2 is, in this way, the only pharmaceutical tested with no toxic effect reported towards methanogenic communities. Nevertheless, in the presence of higher concentrations of EE2, our results showed an inhibitory effect on SMA from acetate and VFA, revealing the susceptibility of the acetoclastic archaea and acetogenic bacteria to this hormone (Table 3.5).

As far as we know, only Hu *et al.* (2018) assessed the effect of DCF directly on acetoclastic and hydrogenotrophic activity. In their work, the tested concentrations were below 1 mg/L and only for concentrations ≥ 0.2 mg/L of DCF was detected inhibition towards acetoclastic communities, with methane production decreasing from 53 to 29 mL with DCF increase from 0.04 to 0.7 mg/L. However, in our work and in a study by Symsaris and colleagues (2015), inhibition of the acetoclastic activity was only detected for higher concentrations. In our study, inhibition of (18 ± 5) % and (38 ± 1) % was only observed at 50 and 100 mg/L of DCF, respectively (Table 3.3) and in the study of Symsaris *et al.* (2015) some inhibition was observed with 100 mg/L, but the IC_{50} was 546 mg/L. Additionally, as observed in our work, acetoclastic archaea were reported as more sensitive to DCF than hydrogenotrophic methanogens (Symsaris *et al.*, 2015; Hu *et al.*, 2018). Results obtained by M. Fountoulakis *et al.* (2004) showed moderate inhibition (30 %) of anaerobic sludge fed with acetate, casein and yeast extract, in the presence of 100 mg/L of DCF, which was similar to the verified in our results at the same concentration of DCF when VFA were used as substrate (Table 3.3). In literature was shown that increasing

concentration of CIP (≥ 0.5 mg/L) caused a significant decrease of the rate of methane production and demonstrated a CIP inhibitory effect at concentrations ≥ 10 mg/L in the methanogenic activity (Liu *et al.,* 2013; Mai *et al.*, 2018). This is in accordance with the results obtained in this work where significant inhibitions of SMA were observed at concentrations equal or higher than 1 mg/L of CIP when VFA were used as substrate (Table 3.2 and Table 7.2 in Appendix III). However, in a work carried out by Zhao *et al.* (2018) with nutrient broth, yeast extract, and glucose, methane production rate was significantly inhibited, 42.9 %, at the lowest concentration tested (0.3 mg/L of CIP), while in this work an inhibition of only (18 ± 10) % with VFA was obtained at a concentration of 0.5 mg/L of CIP and no inhibitions above 40 % were achieved at any of the tested concentrations with VFA (Table 3.2). It should be noticed that the substrates tested in the reported literature and in our work were different, which reflect the sensibility of the different groups of microorganisms. Therefore, bacteria degrading complex polymers and sugars can be more or less sensitive to the presence of the antibiotic than the acetogenic bacteria and methanogenic archaea. In our work, IBP apparently does not reveal a negative effect in the SMA with VFA or H_2/CO_2. The effect of IBP was investigated before towards methanogenic communities under 66 mg/L of IBP with glucose, nutrient broth, and yeast extract as substrates and also with 103 and 206 mg/L of IBP with acetate and propionate as substrate, and no effect was observed (Campbell, 2013; Campbell, 2017).

Overall, the methanogenic communities were most affected by CIP and EE2, followed by DCF and IBP, being the acetoclastic archaea the most sensitive group to the presence of these micropollutants

4. EFFECT OF CNT AND CNT@2%FE ON THE ACTIVITY OF METHANOGENIC COMMUNITIES

4.1 Introduction

Carbon based conductive materials improve the rate of anaerobic reactions by serving as electron shuttles (L. Pereira *et al.*, 2016; J. Zhang & Lu 2016), by stimulating the activity of certain microorganisms (Rotaru *et al.,* 2014a), by promoting the interactions between microorganisms belonging to different species (Y. Liu *et al.,* 2017; Z. Zhao *et al.,* 2016) and by stimulating the metabolism of pure cultures of methanogens (Salvador *et al.,* 2017). For example, Ambuchi and collaborators (2017) showed that iron oxide nanoparticles and MWCNT lead to higher CH_4 production. However, CNT may exhibit strong antimicrobial activity towards bacteria (Ong *et al.*, 2010; Sharma *et al.*, 2015). For example, SWCNT causes irrecoverable damage to the outer membrane of the model bacteria *Escherichia coli* K12, causing the release of the intracellular content (Kang *et al.*, 2007). There are no studies, reporting the inhibitory effect of CNT in methanogenesis, being magnesium oxide, silver nanoparticles, ferrihydrite and carbon black the materials reported to affect negatively the methanogenic activity (Martins *et al.*, 2018).

The use of magnetic carbon nanocomposites prepared by the combination of carbon materials and magnetic nanoparticles, which reveal synergistic properties like catalytic activity, and adsorbent and magnetic properties, opens promising possibilities in the field of adsorption and bioremediation technologies (L. Pereira *et al.*, 2015; Sharma *et al.*, 2015; L. Pereira *et al.*, 2017). L. Pereira and collaborators (2017) conducted experiences with carbon nanotubes, SWCNT and impregnated with 2% of iron (CNT@2%Fe) composites. The high efficiency of the materials on the chemical and biological treatment of a solution containing the azo dye acid Orange 10 (AO10) was proved by the great improvement of the rates (up to 79-fold) and of the yields (≈ 3-times) of the reaction, when compared with the control. Although more research is still needed with other pollutants, this material seems very promising for water treatment technologies (L. Pereira *et al.*, 2015, 2017).

Considering the hypothesis of using CNT and magnetic composites of CNT in anaerobic wastewater treatment, it is important to study the effect of these nanoparticles on the activity of the methanogenic community. Therefore, in this chapter, an anaerobic sludge was incubated with the two CM, CNT and CNT@2%Fe, to investigate the individual effect of these compounds in the SMA.

4.2 Materials and Methods

4.2.1 Experimental setup

The effect of the CNT (MWCNT Nanocyl 3100) and CNT@2%Fe was assessed by measuring the specific methanogenic activity (SMA). The biological sludge used as inoculum in the toxicity tests was the same used in the assays for accessing the effect of pharmaceuticals on the SMA (section 3.2.1). SMA of the anaerobic sludge was determined with of a mixture of volatile fatty acids (10 mM acetate, 10 mM propionate and 5 mM butyrate) as substrate, as described in Appendix I. The description of the assays was already described in section 3.2.1, with the following alterations: 1) assays were performed in bottles of 70 mL with a working volume of 35 mL; 2) after the depletion of the residual substrate by incubating the biomass overnight, the nanomaterials were added at a concentration of 100 mg/L, and then the headspace of the bottles were flushed with a mixture of N_2/CO_2 (80:20 % v/v). Nanomaterials concentrations were selected taking into account a work by L. Pereira *et al.* (2010).

4.2.2 Characteristics of the nanomaterials used

According to the supplier, the MWCNT have an average diameter of 9.5 nm and a length of 1.5 μm. This material was characterized by Tessonnier *et al.*, 2009, as having average inner and outer diameters of 4 and 10 nm, respectively. The carbon purity is superior to 95 % and the main impurities are Fe and Co (0.19 % and 0.07 %, respectively), which are growth catalyst impurities; S (0.14 %), probably due to the purification process; and traces of Al (0.03 %) (Tessonnier *et al.*, 2009; R. A. Pereira *et al.*, 2014). These MWCNT present a pH of zero charge (pH_{pzc}) of 7 and so, will be positively charged when in solutions at pH < pHpzc and negatively charged at pH > pH_{pzc}. These commercial MWCNT were used as support of the metal phase (Fe) in the CNT@2%Fe, prepared by incipient wetness impregnation from aqueous solution of iron(III) nitrate, which is described by L. Pereira *et al.* (2017).

4.2.3 Statistical Analysis

Statistical analysis was performed with the GraphPad software. For comparisons between the effects of nanomaterials in the SMA with the values obtained in their absence, the F test it was used, which allows to compare variances. Then the parametric unpaired t test with a p-value of 0.05 for verifying if there were significant differences between the averages of the SMA with and without nanomaterials.

4.3 Results

The results of the effect of CNT and CNT@2%Fe on the SMA, at the concentration of 100 mg/L, are given in Table 4.1.

Table 4.1 - Methanogenic activity on VFA and stimulation (%), without and with 100 mg/L CNT or CNT@2%Fe.

Nanomaterial	Nanomaterial concentration (mg/L)	SMA ($mLCH_4$@SPT/gVSS.day)	Stimulation (%)
-	0	66.4 ± 0.7	0
CNT	100	85 ± 3	28 ± 4
CNT@2%Fe	100	80 ± 3	20 ± 4

The SMA was stimulated in the presence of the nanomaterials, with SMA increasing from 66.4 ± 0.7 $mLCH_4$@SPT/gVSS.day, without nanomaterial, to 85 ± 3 $mLCH_4$@SPT/gVSS.day, with CNT, and to 80 ± 3 $mLCH_4$@SPT/gVSS.day, with CNT@2%Fe (Table 4.1). This corresponds to an increase in SMA of (28 ± 4) %, with CNT, and (20 ± 4) %, with CNT@2%Fe. These differences in the SMA were significant, with a p value of 0.0003 and 0.0033, respectively to CNT and CNT@2%Fe.

4.4 Discussion

Several studies reported the acceleration of methanogenesis by CM, and the common justification is the facilitation of occurrence of DIET as the main interspecies electron transfer mechanism (Martins *et al.*, 2018). However, there are few evidences for DIET in the majority of these studies. In this work, the presence of CNT and CNT@2%Fe increased the activity of the methanogenic communities, thus no inhibition was observed in none of the conditions tested (Table 4.1). The results obtained are in accordance with the results reported before (Salvador *et al.*, 2017). Stimulation of pure cultures of methanogens by MWCNT was reported and butyrate conversion to methane by a syntrophic culture composed of *Syntrophomonas wolfei* and *Methanospirillum hungatei* was 1.5 times faster in the presence of CNT (5 g/L) (Salvador *et al.,* 2017). These results are in accordance with the results obtained in our study with 0.1 g/L of CNT, since SMA in VFA was enhanced approximately 1.2 times. In another study, the SMA was also stimulated in the presence of CNT@2%Fe, even at a higher extent (unpublished results). The reported SMA stimulation was of 50 % in an anaerobic sludge fed with ethanol (unpublished results), while in this study only 20 % was observed (Table 4.1). However, when using CNT at the same

concentration of our study, unpublished results did not observed stimulation of the SMA with ethanol, while in our study was observed an increase of 28 % in SMA with VFA was observed (Table 4.1). Also, Yan *et al*. (2017) did not observed any effect of CNT (1 g/L) in a batch reactor with anaerobic sludge under mesophilic conditions, using glucose as carbon source. These differences may be caused by the difference in the substrate used, or in the microbial composition of the biomass used, or even in the characteristics and concentrations of the nanomaterials studied, since the different physical and chemical characteristics of CMs and differences in the systems where they are applied, can culminate in different results (Martins *et al*., 2018).

In this way, the application of nanomaterials as a way of improving anaerobic digestion, by SMA stimulation, and participating in pharmaceuticals removal appears to be a good strategy.

5. BIODEGRADATION ASSAYS

5.1 Introduction

Complete degradation of pharmaceutical compounds in conventional WWTP is difficult and to overcome this issue various approaches to remove these compounds are now emerging (Jung *et al.*, 2015). Some studies analysed the removal of pharmaceuticals as DCF, IBP and EE2 by adsorption on CM or AD processes, as for example: Pan and collaborators (2008, 2010) and Joseph *et al.* (2011a, 2011b), which achieved a maximum adsorption efficiency of ≈ 95 – 98 % for EE2. Adsorption of IBP on SWCNT and MWCNT of circa 40 % (Cho *et al.,* 2011), and a maximum adsorption coefficient of 41.4 mg_{DCF}/g_{MWCNT} for DCF on MWCNTs (Sotelo *et al.*, 2012) was previously reported. In what concerns to AD for pharmaceuticals biodegradation, the results vary (Table 5.7, 5.8 and 5.9). For instance, high removal extents of pharmaceuticals were achieved in a study made by Carballa and colleagues (2007): (69 ± 10) %, (41 ± 15) %, and (86 ± 9) %, respectively for DCF (10 μg/L), IBP (10 μg/L) and EE2 (4 μg/L). For EE2, the reported removal percentages are higher than for the remaining studied compounds. For example, complete removal was obtained in a study by Musson *et al.* (2010) (EE2 initial concentration of 62 mg/mL, for 56 days of reaction). Worse results are reported for DCF and IBP. Lahti & Oikari (2011), DCF was not biotransformed, but Sari *et al.* (2013), using enriched cultures, obtained a maximum of 40 % of removal. Musson *et al.* (2010) and Gonzalez-Gil *et al.* (2018) achieved similar removal efficiencies treating a solution with 66 mg/L of IBP and 100 μg/L, respectively, by AD, approximately 28 %. Contrary, in a study by Campbell (2013), with 5 mg/L of IBP, any removal was observed.

Adsorption of these pharmaceuticals to CNT@2%Fe was never study, as well as the combination of AD with CNT, and AD with CNT@2%Fe. Those nanomaterials were only applied in our group in CIP removal (Unpublished results). In biological assays, the presence of CNT and CNT@2%Fe increased the removal percentages of CIP from 72 % (achieved in the absence of nanomaterials) to 98 % and 92 %, respectively. In the abiotic assays, 59 % was obtained in the absence of materials and almost 100 % in their presence, however occurring at lower rates (Unpublished results). Based on the results, the removal of CIP was related to different mechanisms: adsorption on biomass, adsorption on nanomaterials and biodegradation. Those ways may occur separately or in combination, depending on the conditions of the process. Moreover, in a previous study with an azo dye, the electrons from the oxidation of iron in CNT@2%Fe were able to reduce it in the abiotic assays (L. Pereira *et al.,* 2017). The same process may also occur with other compounds like CIP.

In this chapter, biodegradability of DCF, IBP and EE2 will be studied in the presence and absence of CNT or CNT@2%Fe.

5.2 Materials and Methods

5.2.1 Experimental setup for the removal of pharmaceuticals

The biological material used as inoculum in the biodegradation analysis was an anaerobic granular sludge collected from Super Bock Group brewery on September 2017, for the assays with IBP and DCF, and on April 2018, for the assays with EE2.

The biodegradation assays were conducted in 200 mL serum bottles, containing 100 mL of working volume, except for the abiotic conditions in the tests with IBP and EE2, which were performed in 160 mL serum bottles, containing 80 mL of working volume. This work volume was composed by the medium, pharmaceuticals, nanomaterials, inoculum and substrate (a mixture of volatile fatty acids composed by 10 mM acetate, 10 mM propionate and 5 mM butyrate, for stimulation of the syntrophic community). The medium used was a sodium bicarbonate buffer at pH 7.0-7.2, without reducing agent. After biomass addition to the biological test vials, all the bottles were sealed with a butyl rubber and an aluminium capsule, the headspace was flushed with N_2/CO_2 (80:20 % v/v), depressurized and then incubated overnight (37 °C and 110 rpm), for depletion of residual substrate and temperature acclimation. According to each condition, 100 mg/L of nanomaterials (characterization in Section 4.2.1) were added to the respective vials, the headspaces were flushed again with a mixture of N_2/CO_2 (80:20 % v/v), depressurized and then the substrate and pharmaceuticals, in the concentrations indicated in Table 5.1, were added. The concentrations of pharmaceuticals were selected considering the results of Chapter 3. For the assays with DCF, 50 mg/L was assumed as the maximum concentration, since the effects caused as assessed by measuring the SMA, were low. Concentrations of 10 and 5 mg/L were selected for of IBP (solution prepared with water and methanol), and EE2 (solution prepared in ethanol), respectively, since for the superior concentrations, differences between the assays with pharmaceutical and the correspondent organic solvents, indicated a possible contribution of the pharmaceuticals for the final toxic effect (Table 7.5, Table 7.7 and Table 7.8 in Appendix III). Control assays containing the pharmaceutical, CNT or CNT@2%Fe, VFA as carbon source, but without sludge, were performed, to evaluate the effect of the adsorption of the pharmaceuticals (assays). Additionally, a biological control with only VFAs was performed, to estimate biomass activity without the interference of the pharmaceutical and an abiotic control also without pharmaceuticals and without CM (activity negative control). All the assays were performed in triplicate. During the assay, the methane content of all vials was quantified by GC. VFA and ethanol were monitored by High Performance Liquid Chromatography

(HPLC) and pharmaceuticals by Ultra-High-Performance Liquid Chromatography (UHPLC) at the operation times indicated in the Table 5.1. These methodologies are described below.

Table 5.1 – Concentration of pharmaceuticals and organic solvents (used to dissolve IBP and EE2) in the biodegradation assays, and reaction times analysed

Pharmaceutical	[Pharmaceutical] (mg/L)	Organic solvent	Reaction time
DCF	50	No organic solvent used	0, 1, 2, 4, 6, 24 and 48 h 1 month*
IBP	10	73 mM of methanol	0, 1, 2, 4, 6, 8, 24 and 48 h 6 and 13 days
EE2	5	32 mM of ethanol	0, 1, 2, 4, 6, 24 and 48 h

*New substrate addition after 7 and 14 days.

5.2.1.1 Analysis of pharmaceuticals by UHPLC

The concentrations of pharmaceuticals during the time of the process, were monitored by UHPLC in a Shimadzu Nexera XZ, equipped with a diode array detector (SPD-M20A), autosampler (SIL-30AC), degassing (DGU-20A5R), LC-30AD solvent delivery unit, and LC -30AD, and a Labsolutions software. A RP-18 endcapped Purospher Star column (250 mm x 4 mm, 5μm particle size, from MERK, Germany) was used. For the analysis, a volume of 1 mL was collected from the reactors, with a syringe, at the different times of the incubation (Table 5.1), and centrifuged at 15000 rpm during 10 min, to remove the sludge and nanomaterials.

The percentage of removal was calculated applying the Equation 5.1.

$$PR\ (\%) = \frac{C_f - C_0}{C_0} \times 100 \qquad \text{Equation 5.1}$$

Where:

PR = Pharmaceutical removal

C_f = Final concentration of pharmaceutical

C_0 = Initial concentration of pharmaceutical

The UHPLC method used for DCF analysis, was adapted from that described by Freer and collaborators (2014). The mobile phase was composed of 70 % (v/v) of methanol and 30 % (v/v) of 20

mM phosphate buffer (pH 2.5), and the samples were eluted at a flow of 1mL/min, at 30° C, with a total running time of 20 minutes. The detection was performed at 230 nm. A calibration curve ranging from 1.56 to 100 mg/L of DCF was made (Figure 7.1, Appendix VI).

The method used for IBP analysis, was adapted from the method described by Patel and collaborators (2013). The elution was made at a flow rate of 1.1 mL/min, with a gradient made with 15 mM phosphate buffer, pH 3.25 (A) and acetonitrile (B), over 30 minutes, as defined in

Table 5.2. The temperature of the column was set to 35° C. The detection was performed at 230 nm. A calibration curve ranging from 0.625 to 20 mg/L of IBP was made (Figure 7.2, Appendix VI).

Table 5.2 – Gradient of mobile phase for the UHPLC analysis of IBP. A, 15 mM phosphate buffer, pH 3.25 and B, acetonitrile

Time (min)	**0**	**3**	**16**	**17**	**26**	**27**	**30**
% A	80	80	45	35	10	80	80
% B	20	20	55	65	90	20	20

The UHPLC method described by Clara *et al.*, (2004) was adapted for the analysis of EE2. A water – acetonitrile solution (60:40 % v/v) was used as mobile phase. Isocratic elution with a constant flow rate of 1 mL/min was applied, at 25° C. The running time was 30 minutes. EE2 was detected at 225 nm. A calibration curve ranging from 0.313 to 20 mg/L of EE2 was made (Figure 7.3, Appendix VI)

5.2.1.2 Analysis of methane by GC

The GC analysis were performed similarly as described in section 3.2.2. Methane content in the reactors was determined comparing the peak areas of the samples with the one of the standard mixture (40% CH_4), taking into account the mmol quantity of CH_4 in the standard. The volume of CH_4 in the standard was calculated with the Equation 5.2, knowing that 40 % of the volume injected of the sample (0.5 mL) are CH_4, so the CH_4 volume is 0.2 mL

$$PV = nRT \qquad \text{Equation 5.2}$$

Where,

P= pressure (atm), which was 1

V= Volume (mL), which was 0.2

n = number of moles (mmol)

R = gas constant, which is 0.08206

T = temperature (K), which was 310° K

5.2.1.3 Analysis of VFA by HPLC

The substrate monitorization was performed by HPLC (Equipment Jasco, Japan) equipped with an UV detector (Jasco UV 2075 Plus) and a RI detector Jasco RI 4030, an autosampler (Jasco AS 4050), degassing (Jasco DG 2080-53), an oven (Eldex CH-150) and Jasco Chrompass software. An Aminex HPX-87H (300 x 7.8 mm) column from Bio-Rad was used. The temperature of the column in the analysis was 60° C, and the elution was performed with a flow rate of 0.7 mL/min. The mobile phase was a solution of sulfuric acid (5 mM). Calibration curves ranging from 30 to 2500 mg/L of each VFA and ethanol were made (Figure 7.4, Appendix VI). For the analysis, a volume of 1 mL was collected from the reactors, with a syringe, at increasing reaction times (Table 5.1). Samples were then centrifuged at 15000 rpm during 10 min, to remove the sludge and nanomaterials, and the supernatants were filtered with Spartan 13/0.2 RC filters, Whatman 0.2 μm pore size.

5.3 Results

5.3.1 Removal of diclofenac, ibuprofen and 17α-ethinylestradiol

The removal extents of DCF, IBP and EE2 at the different conditions, as evaluated by UHPLC, were calculated and the main results are presented in Table 5.3. For the other conditions there was no removal. The UHPLC chromatograms are available in Appendix VII.

The DCF was highly recalcitrant in all the conditions tested. In biological experiments, removal of 15 % occurred only with VFA and CNT and, surprisingly, in the assay without substrate, also 15 %. In the abiotic assays similar extents of removal were obtained, except for the assay with CNT. For IBP, 29 % of removal was achieved with VFA and CNT, in biological assay, and 40 % in the abiotic control. Relatively to the hormone, removal was obtained in all the biological assays, ranging from circa 40-60%. Better results were found with CM in the presence of VFA: (61 ± 8) % with CNT and (59 ± 7) % with CNT@2%Fe. In the abiotic reactors, removal occurs only in the presence of CM, achieving 100 %.

Table 5.3 - Removal extent (%) of DCF, IBP and EE2 at different conditions after 48 h of reaction. For the conditions not showed the removal was zero

Condition	**Removal (%)**	
	Biological	**Abiotic**
DCF	15 ± 6	10 ± 6
DCF with VFAs	0	15 ± 9
DCF with CNT@2%Fe	0	19 ± 7
DCF with VFA and CNT	15 ± 2	0
IBP with VFA and CNT*	29	40
EE2	43 ± 7	0
EE2 with VFA	42 ± 4	0
EE2 with CNT	48 ± 4	100 ± 1[a]
EE2 with VFA and CNT	61 ± 8	100 ± 1[a]
EE2 with CNT@2%Fe	55 ± 2	100 ± 1[a]
EE2 with VFA and CNT@2%Fe	59 ± 7	100 ± 1[a]

Values with higher standard deviation and negative values were considered as zero.

*Only one replicate was analysed.

[a] - Results observed after 2 h of reaction

5.3.2 Consumption of VFA and CH_4 production in the removal assays of pharmaceutical compounds

Consumption of VFAs, and CH_4 production, was verified by HPLC and GC, respectively, confirming the occurrence of biological activity in the reactors for the biological removal of pharmaceuticals (Table 5.4, 5.5 and 5.6).

Table 5.4 – Concentration of VFA at 0 h (t0) and 48 h (t48) of reaction, and methane produced after 48 h of reaction, at the different conditions

Condition	Acetate (mM)		Propionate (mM)		Butyrate (mM)		CH_4 (mM)
	t0	t48	t0	t48	t0	t48	
DCF with VFA	10 ± 1	4 ± 2	11 ± 1	0	3.5 ± 0.4	2.5 ± 0.1	20 ± 2
DCF with CNT and VFA	10 ± 1	0.2 ± 0.2	10.92 ± 0.05	0	3.36 ± 0.03	2.26 ± 0.03	22 ± 1
DCF with CNT@2%Fe and VFA	9.96 ± 0.07	0.04 ± 0.03	10.68 ± 0.08	0	3.27 ± 0.02	2.24 ± 0.04	22 ± 1
VFA	10.08 ± 0.04	0	10.81 ± 0.04	0	3.31 ± 0.02	1.80 ± 0.01	23 ± 1

For values with higher standard deviation, the value was considered as zero

Table 5.5 – Concentration of VFA at 0 h (t0) and 48 h (t48) of reaction, and methane produced after 48 h of reaction, at the different conditions

Condition	Acetate (mM)		Propionate (mM)		Butyrate (mM)		CH_4 (mM)
	t0	t48	t0	t48	t0	t48	
IBP with VFA	11	1	12	5	3.5	2.7	18 ± 3
IBP with CNT and VFA	nd	nd	nd	nd	nd	nd	16.6 ± 0.9
IBP with CNT@2%Fe and VFA	10	1	11	7	3.3	3.0	19 ± 2
VFA	16	0.1	17	2	5.4	3.0	15.0 ± 0.1

For values with higher standard deviation, the value was considered as zero

nd: not determined

Table 5.6 – Concentration of VFA and of ethanol at 0 h (t0) and 48 h (t48) of reaction, and methane produced after 48 h of reaction, at the different conditions.

Condition	Acetate (mM)		Propionate (mM)		Butyrate (mM)		Ethanol (mM)		CH_4 (mM)
	t0	t48	t0	t48	t0	t48	t0	t48	
EE2	0.12 ± 0.03	3.06 ± 0.03	0	0	0	0	22 ± 3	0	32.9 ± 0.9
EE2 with VFA	11.6 ± 0.1	27 ± 1	10.3 ± 0.4	5 ± 2	3.2 ± 0.1	2.6 ± 0.8	34 ± 8	0	26.2 ± 0.6
EE2 with CNT	1.17 ± 0.04	0.07 ± 0.01	0	0	0	0	27 ± 3	0	34.9 ± 0.9
EE2 with CNT@2%Fe	0.93 ± 0.02	0	0	0	0	0	27 ± 3	0	35.9 ± 0.7
EE2 with CNT and VFA	10.8 ± 0.5	27.38 ± 0.09	11 ± 4	4.4 ± 0.2	3 ± 1	3.07 ± 0.02	33 ± 8	0	32 ± 1
EE2 with CNT@2%Fe and VFA	9.8 ± 0.4	26.5 ± 0.5	9 ± 3	4.0 ± 0.6	2.7 ± 0.8	2.8 ± 0.2	33 ± 7	0	30 ± 2
VFA	10.83 ± 0.00	0.05 ± 0.01	10.98 ± 0.02	0	3.39 ± 0.01	1.95 ± 0.01	0	0	22.8 ± 0.3

For values with higher standard deviation, the value was considered as zero.

5.4 Discussion

The results obtained for the removal of DCF are contradictory. On one hand, in the biological assay performed with CNT and with VFA, DCF removal could be attributed to biotransformation assisted by the nanomaterial, and also to adsorption on anaerobic sludge and on CNT. The similar removal extent, achieved in the biological condition with DCF but without CNT, suggests the adsorption of DCF on the anaerobic sludge. However, the lack of DCF removal in biological experiments with VFA (in the absence of CM) suggests no biotransformation nor adsorption on the sludge. The similar removal percentages obtained in abiotic conditions may be attributed to the fact that the removal percentages are low, of circa 15% and standard deviations are in the same order. In the presence of CNT@2%Fe, besides adsorption on nanomaterial, removal can also occur due to biological reduction of the pharmaceutical compound and due to chemical reduction by the electrons generated from the oxidation of iron, as supported by previous results with the azo dye Acid Orange 10 (Pereira *et al.,* 2017). However, the data obtained in this study (Table 5.3) does not allow to conclude that such mechanism is occurring in the DCF biodegradation assays.

Inconsistent results were also obtained for the IBP removal assays. The removal observed in the abiotic condition with CNT in the presence of substrate, can be due to adsorption on nanomaterial, however, removal in abiotic condition with CNT in absence of substrate would also be expected. Indeed, removal under biological conditions was observed with the same material, but in that case three distinct mechanisms are possible to take place alone or simultaneously: 1) biological activity; 2) adsorption on the biomass and 3) adsorption on the material. Notwithstanding, the final removal percentage in the biological conditions was lower when compared with the obtained under abiotic conditions. In addition, no replicates were analysed, due to equipment problems and so, the discussion is based in the results obtained of only one analysis for condition.

For the removal of EE2, different mechanism may also take place. The results obtained in the assays performed without VFA indicate possible adsorption on biomass. However, the ethanol present in the reactors, used for EE2 dissolution, was also used by the anaerobic microorganisms as substrate. Ethanol oxidation releases electrons that can also be utilized for the biotransformation of EE2. In the reactors containing VFA, and in the absence CM, similar percentages of removal, of circa 40 %, were obtained with adsorption and biological reduction appearing as possible mechanisms. The EE2 removal obtained in the biological tests performed with CNT, (48 ± 4) %, and CNT@%Fe (55 ± 2) %, in the absence of VFA, can be due to adsorption on the biomass, biological activity (as ethanol is present) and to EE2 adsorption on the CM. Removal percentages were increased to circa 60 % with the presence of VFA,

which may be due to the extra electrons generated by VFA oxidation. It is worth noting that ethanol is present in all the assays. Adsorption of EE2 on CM is notorious as in the abiotic assays 100 % of EE2 removal was obtained. The higher values of EE2 removal, in the biological assays containing CM (Table 5.3), indicate that EE2 is possibly adsorbing on the biomass and on CM. Moreover, additional peaks were observed at 48 h of reaction samples by UHPLC analysis, indicating the possible reduction of EE2 (Figure 7.7 in Appendix VII), however further research is necessary.

The VFA results demonstrated that biomass maintained the activity in all biological assays (Table 5.4 to 5.6). Neither the drugs nor the materials had effect on methane production. The presence of ethanol leaded to the production of methane in the tests without VFA (Table 5.6), which supports the possibility of the biodegradation contribution for the removal of EE2, in the tests without VFA.

EE2 and DCF are polycyclic compounds, while IBP is cyclic. Cyclic compounds are more biodegradable than aliphatic polycyclic ones (Musson *et al.*, 2010). However, these compounds present different pK_a which will also influence the removal by adsorption. In this way, considering the pharmaceuticals structure, higher susceptibility of IBP to biodegradation would be expected. Nevertheless, EE2 presents the higher pK_a (10.5) comparing to IBP and DCF pK_a (4.9 and 4.15, respectively). At pH 7, EE2 will be positively charged, while IBP and DCF will have negative charge. Since AS generally presents also negative charge (Jia, *et al.*, 1996), the adsorption between the IBP or DCF and the biomass will be reduced, due to growing repulsion electrostatic interactions and a reduction in $\pi-\pi$ interactions. Additionally, sorption capacity to CM is also pH dependent. The materials tested will have neutral charge at pH 7, due to their pH_{pzc} of 7 (Pereira *et al.,* 2017). Higher adsorption is expected at pH below the pharmaceuticals pK_a (Cho *et al.*, 2011), so higher removal for EE2 due to adsorption would be expected, relatively to the remaining pharmaceuticals studied. This is in accordance with the obtained results.

For polar acidic pharmaceuticals as DCF, biotransformation, direct or co-metabolic, is considered the main removal process (Sari *et al.*, 2013). Electronic withdrawing functional groups of DCF, which causes resistance to anaerobic biodegradation may be one of the reasons for the low values of DCF removal biologically (Cherik *et al.*, 2015). Some authors have obtained biological removal of this compound, however, besides the much lower concentrations used (between 5000 to 5 times lower), acclimatization was made, which allowed the adaptation of the community to the pharmaceutical and the development of removal biochemical systems. The low pK_a influence adsorption capacity at pH 7, which explains the absence of adsorption of this pharmaceutical to biomass in most of the assays.

Data from the literature shows divergent results concerning the adsorption and anaerobic digestion of the studied pharmaceuticals (Table 5.7, 5.8 and 5.9). DCF, IBP and EE2 removal was achieved by some authors, however main differences can be seen in the reaction time, that was usually more extended than in this work, and the used anaerobic sludge was acclimatized, which is a possible explanation for the higher removal achieved relatively to our work (Carballa *et al.*, 2007; Musson *et al.*, 2010; Campbell, 2013; Sari *et al.*, 2013). It should be notice that, removal of DCF with anaerobic sludge not acclimatized, resulted in low removal efficiencies as well (<20%) (Lahti & Oikari, 2011; Gonzalez-Gil *et al.*, 2018). Complete degradation of 504 μg/L and 10,816 μg/L of DCF was achieved in 6 and 25 days of treatment, respectively, using acetate as supplement carbon source in a culture of *Labrys portucalensis* F11, instead of anaerobic sludge (Moreira *et al., 2*018). The conditions in which this study was developed were quite different from the conditions tested in the work reported in thesis since, instead of using complex anaerobic microbial communities, the DCF treatment was performed by using only a bacterial strain and under aerobic conditions. Despite the differences comparing to our work, it should be highlighted the capacity of complete degradation of DCF by a single bacterial strain isolated from the environment. It should be noticed, that *L. portucalensis* F11 was isolated from an industrially polluted site and was already described as having the capacity to degrade various aromatic compounds. Adsorption of this pharmaceutical to materials is also reported in literature, however for higher times of exposure than in this work (from 2 days to 14 days) and on different materials, such as the metal organic framework UiO-66 (Universitetet i Oslo) (Sotelo *et al.*, 2012; Hasan *et al.*, 2016). Variation in materials characteristics, as their mesoporous volume and superficial chemical groups (which influence the material charge), may lead to different removal efficiencies which makes difficult to compare results.

Adsorption of IBP to MWCNT is reported in literature, with similar values of removal as the observed in this work, however the work from literature concerns treatments operated at higher times and lower testing concentration of pharmaceutical was used (Cho *et al.*, 2011) (Table 5.8).

The EE2 removal results presented in this chapter are similar to those obtained by Gonzalez-Gil *et al.* (2018). In their work, the substrate used was the same as the used in our study, however the concentration of EE2 was lower (500 times less EE2), longer periods of reaction were presented and they did not test the influence of nanomaterials (Table 5.9). Higher removal results were obtained in our study even using higher initial concentration and lower reaction periods when compared with other reported studies (Pan *et al.,* 2010; Joseph *et al.,* 2013), with exception of the work performed by Joseph *et al.* (2013) with SWCNT, which achieved similar results.

Table 5.7 – Percentages of removal (R) reported in literature for removal of diclofenac, and the conditions of the treatment

	C_0 (µg/L)	R (%)	Process	Time	pH	Substrate	Reference
DCF	N.A.	41.4 $mg_{pharmaceutical}/g_{MWCNT}$	Adsorption by MWCNT	14 d	3-9	None	(Sotelo *et al.*, 2012)
	1000 to 50,000	189 $mg_{pharmaceutical}/g_{UiO-66}$; adsorption kinetics of 0.014 g/mg.h	Adsorption by Metal-organic framework	N.A.	5.4	None	(Hasan *et al.*, 2016)
	10	80	AD*	SRT 10 d (2 months)	6-7	N.A.	(Carballa *et al.*, 2007)
	10	25-45 (6% sorption)	AD (AS acclimatized)	42 d	7	Glucose and yeast extract	(Sari *et al.*, 2013)
	100	18	AD	4 d	6-7	VFA	(Gonzalez-Gil *et al.*, 2018)
	1000 and 100	0	AD	161 d	7.5	Acetate	(Lahti & Oikari, 2011)
	504	100	Biodegradation with *Labrys portucalensis* F11	6 d	N.A.	Acetate	(Moreira *et al.*, 2018)
	10,816	100	Biodegradation with *Labrys portucalensis* F11	25 d	N.A.	Acetate	(Moreira *et al.*, 2018)
	50,000	19	Adsorption by CNT@2%Fe	2 d	7	None	Our study

C_0- Initial concentration; R- removal; AD – Anaerobic degradation; d – days; N.A: - information not available. SRT – Sludge retention time; *Sludge adapted during the experiment

Table 5.8 - Percentages of removal (R) reported in literature for removal of ibuprofen, and the conditions of the treatment

	C_0 (µg/L)	R (%)	Process	Time	pH	Substrate	Reference
IBP	50 to 2000	40	Adsorption by SWCNT and MWCNT	7 d	$<pK_a$ IBP	None	(Cho *et al.*, 2011)
	10	90	AD	SRT 10 d (2 months)	6-7	N.A.	(Carballa *et al.*, 2007)
	66,000	28	AD	56 d	N.A.	Nutrient solution with sodium bicarbonate	(Musson *et al.*, 2010)
	100	27	AD	4 d	6-7	VFAs	(Gonzalez-Gil *et al.*, 2018)
	5000	2.1	AD	60 d	N.A.	Glucose, nutrient broth and yeast extract	(Campbell, 2013)
	10,000	40	Adsorption by MWCNT	2 d	7	VFA	Our study

C_0- Initial concentration; R- removal; AD – Anaerobic degradation; d – days. N.A. – Information not available; SRT – Sludge retention time

Table 5.9 - Percentages of removal (R) reported in literature for removal of 17α-ethinylestradiol, and the conditions of the treatment

	C_0 (μg/L)	R (%)	Process	Time	pH	Substrate	Reference
EE2	3300	29	Adsroption by SWCNT	17.7 h	7	None	(Pan *et al.*, 2010)
	3300	69.6	Adsorption by MWCNT	7.7 h	7	None	(Pan *et al.*, 2010)
	296	91.4	Adsorption by MWCNT	4 h	5	None	(Joseph *et al.*, 2013)
	296	>99	Adsorption by SWCNT	4 h	5	None	(Joseph *et al.*, 2013)
	4	86 ± 9	AD *	SRT 10 d (2 months)	6-7	N.A.	(Carballa *et al.*, 2007)
	62,000	100	AD	56 d	N.A.	Nutrient solution	(Musson *et al.*, 2010)
	10	55 ± 29	AD	4 d	6-7	VFA	(Gonzalez-Gil *et al.*, 2018)
	5000	100	Adsorption by MWCNT or CNT@2%Fe	2 h	7	Absence or presence of VFA	Our study
	5000	60	AD with CNT or CNT@2%Fe	2 d	7	VFA	Our study

C_0- Initial concentration; R- removal; AD – Anaerobic degradation; d – days: N.A. – Information not available. SRT – Sludge retention time;

*Sludge adapted during the experiment

.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

The pharmaceuticals tested, ciprofloxacin (CIP), diclofenac (DCF), ibuprofen (IBP) and 17α-ethinylestradiol (EE2) affected the methanogenic activity of the anaerobic sludge used. The acetoclastic methanogens were the most sensitive, being affected by all pharmaceuticals. On the other hand, the hydrogenotrophic archaea were almost not affected and the acetogenic bacteria was affected by all CIP, DCF and EE2, but less than the acetoclastic. Overall, the methanogenic communities were most affected by ciprofloxacin and 17α-ethinylestradiol, followed by diclofenac and ibuprofen. Carbon nanomaterials did not presented toxicity towards the anaerobic sludge, since it even stimulated the methanogenic activity, primarily by CNT.

The results for biotransformation did not reveal reduction of DCF or IBP. However, the interpretation of these results was difficult due to the inconsistent results obtained. The application of carbon nanomaterials in the biological treatment of EE2 increased the removal of this hormone from approximately 42 % to circa 60 %. Additionally, complete removal of EE2 was achieved in abiotic assays with carbon nanomaterials. No differences in the efficiency of this treatment process was obtained between CNT or CNT@2%Fe. Mostly, the removal efficiencies in the different conditions were obtained in 2 hours of treatment. In this way, the application of nanomaterials to remove EE2 appears to be a good strategy, since it is a fast and efficient process. However, no conclusion was possible to make about the mechanisms of EE2 removal and further research is needed.

At present, as far as we know, there are no papers that study the anaerobic biotransformation of these pharmaceuticals in the presence of CNT and CNT@2%Fe. Anyway, the results were not conclusive and further studies must be conducted, for instance with acclimatized biomass and with extended incubation times. Further, tailoring the materials, as for example modifying the chemistry of the surface, may also be a strategy. Altering the surface chemistry of materials may allow their functionalisation for specific compounds, because of their amphoteric character. As oxidation of iron on nanomaterials can also give electrons, which may be used for the reduction of pollutants in anaerobic digestion, increasing its concentration in nanocomposites may also influence the efficiency of the process. New biodegradation assays with higher EE2 concentrations could be made. An extra control assay with a biomass inhibitor could also be performed, in order to try to distinguish the EE2 removal through adsorption from the EE2 biotransformation. In the case of EE2, to which removal was observed, a toxicity of the compounds after this treatment in a model organism, as *Vibrio fisheri*, could be assessed. Attempt for the identification of possibly by-products, formed during EE2 biotransformation, is also important for predicting the mechanism as well as the impact, relating them to toxicity results. Moreover, assays with wastewater and contaminated sludge, special sludge with pharmaceutical compounds (sludge already adapted to them),

but where other compounds are also present, should be made, to assess a more realistic removal efficiency of the process.

REFERENCES

Adekunle, K. F., & Okolie, J. A. (2015). A Review of Biochemical Process of Anaerobic Digestion. *Advances in Bioscience and Biotechnology*, *6*(3), 205–212. http://doi.org/10.4236/abb.2015.63020

Ahn, H. K., Smith, M. C., Kondrad, S. L., & White, J. W. (2010). Evaluation of biogas production potential by dry anaerobic digestion of switchgrass-animal manure mixtures. *Applied Biochemistry and Biotechnology*, *160*(4), 965–975. http://doi.org/10.1007/s12010-009-8624-x

Ambuchi, J. J., Zhang, Z., Shan, L., Liang, D., Zhang, P., & Feng, Y. (2017). Response of anaerobic granular sludge to iron oxide nanoparticles and multi-wall carbon nanotubes during beet sugar industrial wastewater treatment. *Water Research*, *117*, 87–94. http://doi.org/10.1016/j.watres.2017.03.050

Amezquita-Garcia, H. J., Rangel-Mendez, J. R., Cervantes, F. J., & Razo-Flores, E. (2016). Activated carbon fibers with redox-active functionalities improves the continuous anaerobic biotransformation of 4-nitrophenol. *Chemical Engineering Journal*, *286*, 208–215. http://doi.org/10.1016/j.cej.2015.10.085

Butkovskyi, A., Hernandez Leal, L., Rijnaarts, H. H. M., & Zeeman, G. (2015). Fate of pharmaceuticals in full-scale source separated sanitation system. *Water Research*, *85*, 384–392. http://doi.org/10.1016/j.watres.2015.08.045

Camargo, P. H. C., Satyanarayana, K. G., & Wypych, F. (2009). Nanocomposites: Synthesis, Structure, Properties and New Application Opportunities. *Materials Research*, *12*(1), 1–39. http://doi.org/10.1590/S1516-14392009000100002

Campbell, A. J. (2013). The behaviour of pharmaceuticals in anaerobic digester sludge, (February), 238. Retrieved from https://researchportal.port.ac.uk/portal/en/organisations/university-of-portsmouth(adc9c7e1-f6d1-4d2e-811f-da39063ee035)/theses.html?page=24

Campbell, J. K. (2017). *Biotransformation of Atenolol and Ibuprofen under Methanogenic Conditions and their Influence on Gas Production and the Microbial Community (MSc thesis)*. The State University of New Jersey, New Brunswick, New Jersey. http://doi.org/10.7282/T3RR21PN

Carballa, M., Omil, F., Ternes, T., & Lema, J. M. (2007). Fate of pharmaceutical and personal care products (PPCPs) during anaerobic digestion of sewage sludge. *Water Research*, *41*(10), 2139–2150. http://doi.org/10.1016/j.watres.2007.02.012

Carvalho, P. N., Pirra, A., Basto, M. C. P., & Almeida, C. M. R. (2013). Activated sludge systems removal efficiency of veterinary pharmaceuticals from slaughterhouse wastewater. *Environmental Science and Pollution Research*, *20*(12), 8790–8800. http://doi.org/10.1007/s11356-013-1867-7

Cetecioglu, Z., Ince, B., & Azman, S. (2013). Determination of Anaerobic and Anoxic Biodegradation Capacity of Sulfamethoxasole and the Effects on Mixed Microbial Culture. In *Biodegradation - Engineering and Technology* (pp. 223–249).

Chen, C., Hu, J., Shao, D., Li, J., & Wang, X. (2009). Adsorption behavior of multiwall carbon nanotube/iron oxide magnetic composites for Ni(II) and Sr(II). *Journal of Hazardous Materials*, *164*(2–3), 923–928. http://doi.org/10.1016/j.jhazmat.2008.08.089

Cherik, D., Benali, M., & Louhab, K. (2015). Occurrence, ecotoxicology, removal of diclofenac by adsorption on activated carbon and biodegradation and its effect on bacterial community: A review. *World Scientific News*, *16*, 116–144. Retrieved from http://psjd.icm.edu.pl/psjd/element/bwmeta1.element.psjd-c2b1b07d-eecb-4752-80ab-67e57240d9db

Cho, H. H., Huang, H., & Schwab, K. (2011). Effects of solution chemistry on the adsorption of ibuprofen and triclosan onto carbon nanotubes. *Langmuir*, *27*(21), 12960–12967. http://doi.org/10.1021/la202459g

Clara, M., Strenn, B., Saracevic, E., & Kreuzinger, N. (2004). Adsorption of bisphenol-A, 17β-estradiole and 17α-ethinylestradiole to sewage sludge. *Chemosphere*, *56*(9), 843–851. http://doi.org/10.1016/j.chemosphere.2004.04.048

Coates, J. D., Coughlan, M. F., & Colleran, E. (1996). Simple method for the measurement of the hydrogenotrophic methanogenic activity of anaerobic sludges. *Journal of Microbiological Methods*, *26*(3), 237–246. http://doi.org/10.1016/0167-7012(96)00915-3

Colleran, E., Concannon, F., Golden, T., Geoghegan, F., Crumlish, B., Killilea, E., ... Coates, J. (1992). Use of methanogenic activity tests to characterize anaerobic sludges, screen for anaerobic biodegradability and determine toxicity thresholds against individual anaerobic trophic. *Water Science and Technology*, *25*(7), 31–40.

Donohoe, M. (2003). Causes and health consequences of environmental degradation and social injustice. *Social Science & Medicine*, *56*, 573–587.

Fountoulakis, M., Drillia, P., Stamatelatou, K., & Lyberatos, G. (2004). Tonic effect of pharmaceuticals on methanogienesis. *Water Science and Technology*, *50*(5), 335–340.

Fountoulakis, M. S., Stamatelatou, K., & Lyberatos, G. (2008). The effect of pharmaceuticals on the kinetics of methanogenesis and acetogenesis. *Bioresource Technology*, *99*(15), 7083–7090. http://doi.org/10.1016/j.biortech.2008.01.008

Freer, J., Baeza, C., Contreras, D., Soto, C., Corrales, S., & Moreno, N. (2014). Simultaneous determination of atrazine and sodium diclofenac in natural water by spectrophotometric approaches. *Journal of the Chilean Chemical Society*, *59*(3), 2632–2635. http://doi.org/10.4067/S0717-97072014000300024

Gadelle, D., Filée, J., Buhler, C., & Forterre, P. (2003). Phylogenomics of type II DNA topoisomerases. *BioEssays*, *25*(3), 232–242. http://doi.org/10.1002/bies.10245

Gonzalez-Gil, L., Mauricio-Iglesias, M., Serrano, D., Lema, J. M., & Carballa, M. (2018). Role of methanogenesis on the biotransformation of organic micropollutants during anaerobic digestion. *Science of the Total Environment*, *622–623*, 459–466. http://doi.org/10.1016/j.scitotenv.2017.12.004

Hartmann, J., Bartels, P., Mau, U., Witter, M., Tümpling, W. v., Hofmann, J., & Nietzschmann, E. (2008). Degradation of the drug diclofenac in water by sonolysis in presence of catalysts. *Chemosphere*, *70*(3), 453–461. http://doi.org/10.1016/j.chemosphere.2007.06.063

Hasan, Z., Khan, N. A., & Jhung, S. H. (2016). Adsorptive removal of diclofenac sodium from water with Zr-based metal–organic frameworks. *Chemical Engineering Journal*, *284*, 1406–1413. http://doi.org/10.1016/j.cej.2015.08.087

Hirsch, A. (2002). Functionalization of single-walled carbon nanotubes. *Angewandte Chemie - International Edition*, *41*(11), 1853–1859. http://doi.org/10.1002/1521-3773(20020603)41:11<1853::AID-ANIE1853>3.0.CO;2-N

Holmes, D. E., & Smith, J. A. (2016). Biologically Produced Methane as a Renewable Energy Source. In *Advances in Applied Microbiology, 97* (p. 242).

Hom-Diaz, A., Baldi, F., Blánquez, P., Lombardi, L., Martín-González, L., & Vicent, T. (2016). Exhausted fungal biomass as a feedstock for increasing methane production during the anaerobic digestion of organic wastes. *Waste and Biomass Valorization*, *7*(2), 307–315. http://doi.org/10.1007/s12649-015-9450-8

Hu, J., Zhao, J., Wang, D., Li, X., Zhang, D., Xu, Q., ... Zeng, G. (2018). Effect of diclofenac on the production of volatile fatty acids from anaerobic fermentation of waste activated sludge. *Bioresource Technology*, *254*, 7–15. http://doi.org/10.1016/j.biortech.2018.01.059

Ji, J. Y., Xing, Y. J., Ma, Z. T., Zhang, M., & Zheng, P. (2013). Acute toxicity of pharmaceutical wastewaters containing antibiotics to anaerobic digestion treatment. *Chemosphere*, *91*(8), 1094–1098. http://doi.org/10.1016/j.chemosphere.2013.01.009

Ji, L., Chen, W., Zheng, S., Xu, Z., & Zhu, D. (2009). Adsorption of sulfonamide antibiotics to multiwalled carbon nanotubes. *Langmuir*, *25*(19), 11608–11613. http://doi.org/10.1021/la9015838

Jia, X. S., Fang, H. H. P., & Furumai, H. (1996). Surface charge and extracellular polymer of sludge in

the anaerobic degradation process. *Water Science and Technology*, *34*(5–6–6 pt 3), 309–316. http://doi.org/10.1016/0273-1223(96)00660-9

Jorgenson, A. K. (2003). Footprint consumption and environmental degradation : A Cross-National Analysis. *University of California Press*, *50*(3), 374–394.

Joseph, L., Boateng, L. K., Flora, J. R. V., Park, Y. G., Son, A., Badawy, M., & Yoon, Y. (2013). Removal of bisphenol A and 17α-ethinylestradiol by combined coagulation and adsorption using carbon nanomaterials and powdered activated carbon. *Separation and Purification Technology*, *107*, 37–47. http://doi.org/10.1016/j.seppur.2013.01.012

Joseph, L., Heo, J., Park, Y. G., Flora, J. R. V., & Yoon, Y. (2011a). Adsorption of bisphenol A and 17α-ethinylestradiol on single walled carbon nanotubes from seawater and brackish water. *Desalination*, *281*(1), 68–74. http://doi.org/10.1016/j.desal.2011.07.044

Joseph, L., Zaib, Q., Khan, I. A., Berge, N. D., Park, Y. G., Saleh, N. B., & Yoon, Y. (2011b). Removal of bisphenol A and 17α-ethinylestradiol from landfill leachate using single-walled carbon nanotubes. *Water Research*, *45*(13), 4056–4068. http://doi.org/10.1016/j.watres.2011.05.015

Jung, C., Son, A., Her, N., Zoh, K. D., Cho, J., & Yoon, Y. (2015). Removal of endocrine disrupting compounds, pharmaceuticals, and personal care products in water using carbon nanotubes: A review. *Journal of Industrial and Engineering Chemistry*, *27*, 1–11. http://doi.org/10.1016/j.jiec.2014.12.035

Kang, S., Pinault, M., Pfefferle, L. D., & Elimelech, M. (2007). Single-walled carbon nanotubes exhibit strong antimicrobial activity. *Langmuir*, *23*(17), 8670–8673. http://doi.org/10.1021/la701067r

Lahti, M., & Oikari, A. (2011). Microbial transformation of pharmaceuticals naproxen, bisoprolol, and diclofenac in aerobic and anaerobic environments. *Archives of Environmental Contamination and Toxicology*, *61*(2), 202–210. http://doi.org/10.1007/s00244-010-9622-2

Langenhoff, A., Inderfurth, N., Veuskens, T., Schraa, G., Blokland, M., Kujawa-Roeleveld, K., & Rijnaarts, H. (2013). Microbial removal of the pharmaceutical compounds ibuprofen and diclofenac from wastewater. *BioMed Research International*, *2013*. http://doi.org/10.1155/2013/325806

Lettinga, G., Lier, J. B. Van, Buuren, J. C. L. Van, & Zeeman, G. (2001). Sustainable development in pollution control and the role of anaerobic treatment. *Water Science and Technology*, *44*(6), 181–188.

Li, L. L., Tong, Z. H., Fang, C. Y., Chu, J., & Yu, H. Q. (2015). Response of anaerobic granular sludge to single-wall carbon nanotube exposure. *Water Research*, *70*, 1–8. http://doi.org/10.1016/j.watres.2014.11.042

Liao, X., Li, B., Zou, R., Dai, Y., Xie, S., & Yuan, B. (2016). Biodegradation of antibiotic ciprofloxacin: pathways, influential factors, and bacterial community structure. *Environmental Science and Pollution Research*, *23*(8), 7911–7918. http://doi.org/10.1007/s11356-016-6054-1

Lier, J. B. Van, Mahmoud, N., & Zeeman, G. (2008). *Anaerobic wastewater treatment*. *biological wastewater treatment : Principles, modelling and design*. http://doi.org/10.1021/es00154a002

Lijó, L., González-garcía, S., Bacenetti, J., & Moreira, M. T. (2017). The environmental effect of substituting energy crops for food waste as feedstock for biogas production. *Energy*. http://doi.org/10.1016/j.energy.2017.04.137

Liu, Y., Zhang, Y., Zhao, Z., Ngo, H. H., Guo, W., Zhou, J., ... Ni, B. J. (2017). A modeling approach to direct interspecies electron transfer process in anaerobic transformation of ethanol to methane. *Environmental Science and Pollution Research*, *24*(1), 855–863. http://doi.org/10.1007/s11356-016-7776-9

Liu, Z., Sun, P., Pavlostathis, S. G., Zhou, X., & Zhang, Y. (2013). Inhibitory effects and biotransformation potential of ciprofloxacin under anoxic/anaerobic conditions. *Bioresource Technology*, *150*, 28–35. http://doi.org/10.1016/j.biortech.2013.09.125

Lovley, D. R. (2008). The microbe electric: conversion of organic matter to electricity. *Current Opinion in*

Biotechnology, *19*(6), 564–571. http://doi.org/10.1016/j.copbio.2008.10.005

Lovley, D. R. (2011). Reach out and touch someone: Potential impact of DIET (direct interspecies energy transfer) on anaerobic biogeochemistry, bioremediation, and bioenergy. *Reviews in Environmental Science and Biotechnology*, *10*(2), 101–105. http://doi.org/10.1007/s11157-011-9236-9

Lu, M., Niu, X., Liu, W., Zhang, J., Wang, J., Yang, J. & Yang, Z. (2016). Biogas generation in anaerobic wastewater treatment under tetracycline antibiotic pressure. *Scientific Reports*, *6*, 28336. http://doi.org/10.1038/srep28336

Mai, D. T., Stuckey, D. C., & Oh, S. (2018). Effect of ciprofloxacin on methane production and anaerobic microbial community. *Bioresource Technology*, *261*, 240–248. http://doi.org/10.1016/j.biortech.2018.04.009

Martins, G., Salvador, A. F., Pereira, L., & Alves, M. M. (2018). Methane Production and Conductive Materials: A Critical Review. *Environmental Science & Technology*, *52*(18), 10241–10253. http://doi.org/10.1021/acs.est.8b01913

McInerney, M. J., Struchtemeyer, C. G., Sieber, J., Mouttaki, H., Stams, A. J. M., Schink, B., ... Gunsalus, R. P. (2008). Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. *Annals of the New York Academy of Sciences*, *1125*, 58–72. http://doi.org/10.1196/annals.1419.005

Meslé, M., Dromart, G., & Oger, P. (2013). Microbial methanogenesis in subsurface oil and coal. *Research in Microbiology*, *164*(9), 959–972. http://doi.org/10.1016/j.resmic.2013.07.004

Moreira, I. S., Bessa, V. S., Castro, P. M. L., Murgolo, S., Piccirillo, C., & Mascolo, G. (2018). Ecotoxicology and Environmental Safety Biodegradation of Diclofenac by the bacterial strain *Labrys portucalensis* F11. *Ecotoxicology and Environmental Safety*, *152*, 104–113. http://doi.org/10.1016/j.ecoenv.2018.01.040

Morita, M., Malvankar, N. S., Franks, A. E., Summers, Z. M., Giloteaux, L., Rotaru, A. E., ... Lovley, D. R. (2011). Potential for Direct Interspecies Electron Transfer in Methanogenic Wastewater Digester Aggregates, *2*(4), 5–7. http://doi.org/10.1128/mBio.00159-11.Editor

Morris, B. E. L., Henneberger, R., Huber, H., & Moissl-Eichinger, C. (2013). Microbial syntrophy: Interaction for the common good. *FEMS Microbiology Reviews*, *37*(3), 384–406. http://doi.org/10.1111/1574-6976.12019

Musson, S. E., Campo, P., Tolaymat, T., Suidan, M., & Townsend, T. G. (2010). Assessment of the anaerobic degradation of six active pharmaceutical ingredients. *Science of the Total Environment*, *408*(9), 2068–2074. http://doi.org/10.1016/j.scitotenv.2009.11.042

NPCS Board. (2013). Ibuprofen. In *Investment Opportunity in Drugs & Pharmaceutical Projects* (2013th ed., pp. 98–117). Delhi: NIIR Project Consultancy Services.

Nunes, B. (2010). Fármacos no ambiente: implicações ecotoxicológicas. *Revista Captar: Ciência e Ambiente Para Todos*, *2*(1), 9–20. Retrieved from http://revistas.ua.pt/index.php/captar/article/view/2724

Oliveira, Luiz C A; Rios, Rachel V R A; Fabris, J D; Garg, V; Sapag, Karim Lago, R. M. (2002). Activated carbon / iron oxide magnetic composites for the adsorption of contaminants in water. *Carbon*, *40*, 2177–2183.

Ong, Y. T., Ahmad, A. L., Hussein, S., Zein, S., & Tan, S. H. (2010). A review on carbon nanotubes in an environmental protection and gree engineering perspective. *Brazilian Journal of Chemical Engineering*, *27*(02), 227–242. http://doi.org/10.1590/S0104-66322010000200002

Pan, B., Lin, D., Mashayekhi, H., & Xing, B. (2008). Adsorption and hysteresis of bisphenol A and 17alpha-ethinyl estradiol on carbon nanomaterials. *Environmental Science & Technology*, *42*(15), 5480–5485. http://doi.org/10.1021/es8001184

Pan, B., Sun, K., & Xing, B. (2010). Adsorption kinetics of 17α-ethinylestradiol and bisphenol A on carbon nanomaterials. *Journal of Soils and Sediments*, *10*(5), 845–854. http://doi.org/10.1007/s11368-

009-0185-7

Panayotou, T. (1993). *Empirical Tests and Policy Analysis of Environmental Degradation at Different Stages of Economic Development by*. Geneva: International Labour Office.

Park, C., Lee, C., Kim, S., Chen, Y., & Chase, H. A. (2005). Upgrading of anaerobic digestion by incorporating two different hydrolysis processes. *Journal of Bioscience and Bioengineering*, *100*(2), 164–167. http://doi.org/10.1263/jbb.100.164

Patel, P. N., Samanthula, G., Shrigod, V., Modh, S. C., & Chaudhari, J. R. (2013). RP-HPLC Method for Determination of Several NSAIDs and Their Combination Drugs. *Hindawi Publishing Corporation Chromatography Research International*, *2013*, 1–13. http://doi.org/10.1155/2013/242868

Pereira, A. M. P. T., Silva, L. J. G., Meisel, L. M., Lino, C. M., & Pena, A. (2015). Environmental impact of pharmaceuticals from Portuguese wastewaters: Geographical and seasonal occurrence, removal and risk assessment. *Environmental Research*, *136*, 108–119. http://doi.org/10.1016/j.envres.2014.09.041

Pereira, L., Dias, P., Soares, O. S. G. P., Ramalho, P. S. F., Pereira, M. F. R., & Alves, M. M. (2017). Synthesis, characterization and application of magnetic carbon materials as electron shuttles for the biological and chemical reduction of the azo dye Acid Orange 10. *Applied Catalysis B: Environmental*, *212*, 175–184. http://doi.org/10.1016/j.apcatb.2017.04.060

Pereira, L., Mehboob, F., Stams, A. J. M., Mota, M. M., Rijnaarts, H. H. M., & Alves, M. M. (2015). Metallic nanoparticles: microbial synthesis and unique properties for biotechnological applications, bioavailability and biotransformation. *Critical Reviews in Biotechnology*, *35*(1), 114–128. http://doi.org/10.3109/07388551.2013.819484

Pereira, L., Pereira, R., Pereira, M. F. R., & Alves, M. M. (2016). Effect of different carbon materials as electron shuttles in the anaerobic biotransformation of nitroanilines. *Biotechnology and Bioengineering*, *113*(6), 1194–1202. http://doi.org/10.1002/bit.25896

Pereira, L., Pereira, R., Pereira, M. F. R., van der Zee, F. P., Cervantes, F. J., & Alves, M. M. (2010). Thermal modification of activated carbon surface chemistry improves its capacity as redox mediator for azo dye reduction. *Journal of Hazardous Materials*, *183*(1–3), 931–939. http://doi.org/10.1016/j.jhazmat.2010.08.005

Pereira, R. A., Pereira, M. F. R., Alves, M. M., & Pereira, L. (2014). Carbon based materials as novel redox mediators for dye wastewater biodegradation. *Applied Catalysis B: Environmental*, *144*, 713–720. http://doi.org/10.1016/j.apcatb.2013.07.009

Quero-Pastor, M. J., Garrido-Perez, M. C., Acevedo, A., & Quiroga, J. M. (2014). Ozonation of ibuprofen: A degradation and toxicity study. *Science of the Total Environment*, *466–467*, 957–964. http://doi.org/10.1016/j.scitotenv.2013.07.067

Ribeiro, A. R., Carvalho, M. F., Afonso, C. M. M., Tiritan, M. E., & Castro, P. M. L. (2010). Microbial degradation of 17β-estradiol and 17α-ethinylestradiol followed by a validated HPLC-DAD method. *Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes*, *45*(4), 265–273. http://doi.org/10.1080/03601231003704523

Rodrigues, A. (2013). *Resíduos farmacêuticos no ambiente*. Instituto Superior de Ciências da Saúde Egas Moniz. Retrieved from http://hdl.handle.net/10400.26/14123

Rotaru, A.-E., Shrestha, P. M., Liu, F., Shrestha, M., Shrestha, D., Embree, M., … Lovley, D. R. (2014a). A new model for electron flow during anaerobic digestion: direct interspecies electron transfer to *Methanosaeta* for the reduction of carbon dioxide to methane. *Energy & Environmental Science*, *7*(1), 408–415. http://doi.org/10.1039/C3EE42189A

Rotaru, A. E., Shrestha, P. M., Liu, F., Markovaite, B., Chen, S., Nevin, K. P., & Lovley, D. R. (2014b). Direct interspecies electron transfer between *Geobacter metallireducens* and *Methanosarcina barkeri*. *Applied and Environmental Microbiology*, *80*(15), 4599–4605. http://doi.org/10.1128/AEM.00895-14

Salvador, A. F., Martins, G., Melle-Franco, M., Serpa, R., Stams, A. J. M., Cavaleiro, A. J., ... Alves, M. M. (2017). Carbon nanotubes accelerate methane production in pure cultures of methanogens and in a syntrophic coculture. *Environmental Microbiology*, *(in press)*. http://doi.org/10.1111/1462-2920.

Santos, L., Teixeira, D., Jacob, R., Amaral, M., & Lange, L. (2014). Evaluation of the aerobic and anaerobic biodegradability of the antibiotic norfloxacin. *Water Science and Technology*, *70*(2), 265–271. http://doi.org/10.2166/wst.2014.214

Sari, S., Özdemir, G., Topuz, E., Aydın, E., & Tas, D. O. (2013). Assessment of Biodegradability of Diclofenac under Methanogenic Conditions. *13th World Congress on Anaerobic Digestion - Recovering (Bio) Resources for the World.*, June 25-28.

Sayes, C. M., Liang, F., Hudson, J. L., Mendez, J., Guo, W., Beach, J. M., ... Colvin, V. L. (2006). Functionalization density dependence of single-walled carbon nanotubes cytotoxicity in vitro. *Toxicology Letters*, *161*(2), 135–142. http://doi.org/10.1016/j.toxlet.2005.08.011

Schink, B. (1997). Energetics of syntrophic cooperation in methanogenic degradation. *Microbiology and Molecular Biology Reviews : MMBR*, *61*(2), 262–280. http://doi.org/1092-2172/97/$04.0010

Schon, M. (2009). *Numerical Modelling of Anaerobic Digestion Processes in Agricultural Biogas Plants Dissertation Anaerobic Digestion Processes in Agricultural Biogas Plants (PhD Thesis)*. Books on demand, Innsbruck.

Senghor, A., Dioh, R. M. N., Müller, C., & Youm, I. (2016). Cereal crops for biogas production : A review of possible impact of elevated. *Renewable and Sustainable Energy Reviews*, 1–7. http://doi.org/10.1016/j.rser.2016.12.082

Sharma, V. K., McDonald, T. J., Kim, H., & Garg, V. K. (2015). Magnetic graphene-carbon nanotube iron nanocomposites as adsorbents and antibacterial agents for water purification. *Advances in Colloid and Interface Science*, *225*, 229–240. http://doi.org/10.1016/j.cis.2015.10.006

Shrestha, P. M., & Rotaru, A. E. (2014). Plugging in or going wireless: Strategies for interspecies electron transfer. *Frontiers in Microbiology*, *5*(237), 1–8. http://doi.org/10.3389/fmicb.2014.00237

Sotelo, J. L., Rodríguez, A. R., Mateos, M. M., Hernández, S. D., Torrellas, S. A., & Rodríguez, J. G. (2012). Adsorption of pharmaceutical compounds and an endocrine disruptor from aqueous solutions by carbon materials. *Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes*, *47*(7), 640–652. http://doi.org/10.1080/03601234.2012.668462

Sousa, I. E. S. (2015). *Métodos de eliminação de fármacos e seus metabolitos ao nível das Estações de Tratamento de Águas Residuais*. Universidade Fernando Pessoa.

Stams, A. (1994). Metabolic Interactions Between Anaerobic-Bacteria in Methanogenic Environments. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, *66*(1–3), 271–294. http://doi.org/10.1007/BF00871644

Stern, D. I., Common, M. S., & Barbier, E. B. (1996). Economic Growth and Environmental Degradation : The Environmental Kuznets Curve and Sustainable Development. *World Development*, *24*(7), 1151–1560.

Sträuber, H., Lucas, R., & Kleinsteuber, S. (2016). Metabolic and microbial community dynamics during the anaerobic digestion of maize silage in a two-phase process. *Applied Microbiology and Biotechnology*, *100*(1), 479–491. http://doi.org/10.1007/s00253-015-6996-0

Summers, Z. M., Fogarty, H. E., Leang, C., Franks, A. E., Malvankar, N. S., & Lovley, D. R. (2010). Direct Exchange of Electrons Within Aggregates of an Evolved Syntrophic Coculture of Anaerobic Bacteria. *Science*, *330*(6009), 1413–1415. http://doi.org/10.1126/science.1196526

Symsaris, E. C., Fotidis, I. A., Stasinakis, A. S., & Angelidaki, I. (2015). Effects of triclosan, diclofenac, and nonylphenol on mesophilic and thermophilic methanogenic activity and on the methanogenic communities. *Journal of Hazardous Materials*, *291*, 45–51.

http://doi.org/10.1016/j.jhazmat.2015.03.002
Tessonnier, J. P., Rosenthal, D., Hansen, T. W., Hess, C., Schuster, M. E., Blume, R., ... Schlögl, R. (2009). Analysis of the structure and chemical properties of some commercial carbon nanostructures. *Carbon*, *47*(7), 1779–1798. http://doi.org/10.1016/j.carbon.2009.02.032
Thiele, J. H., & Zeikus, J. G. (1988). Control of Interspecies Electron Flow during Anaerobic Digestion: Significance of Formate Transfer versus Hydrogen Transfer during Syntrophic Methanogenesis in Flocs. *Applied and Environmental Microbiology*, *54*(1), 20–29. http://doi.org/0099-2240/88/010010-10$02.00/0
Tian, T., Qiao, S., Li, X., Zhang, M., & Zhou, J. (2017). *Nano-graphene induced positive effects on methanogenesis in anaerobic digestion*. *Bioresource Technology* (Vol. 224). http://doi.org/10.1016/j.biortech.2016.10.058
Tian, Y., Gao, B., Chen, H., Wang, Y., & Li, H. (2013). Interactions between carbon nanotubes and sulfonamide antibiotics in aqueous solutions under various physicochemical conditions. *Journal of Environmental Science and Health, Part A*, *48*(9), 1136–1144. http://doi.org/10.1080/10934529.2013.774670
US EPA. (2009). Targeted National Sewage Sludge Survey Overview Report. *United States Environmental Protection Agency Office of Water*, Washington DC, USA.
Vidu, R., Rahman, M., Mahmoudi, M., Enachescu, M., Poteca, T. D., & Opris, I. (2014). Nanostructures: a platform for brain repair and augmentation. *Frontiers in Systems Neuroscience*, *8*(91). http://doi.org/10.3389/fnsys.2014.00091
Weiland, P. (2010). Biogas production : current state and perspectives. *Applied Microbiology and Biotechnology*, *85*, 849–860. http://doi.org/10.1007/s00253-009-2246-7
Xie, X.-L., Mai, Y.-W., & Zhou, X.-P. (2005). Dispersion and alignment of carbon nanotubes in polymer matrix: A review. *Materials Science and Engineering R: Reports*, *49*(4), 89–112. http://doi.org/10.1016/j.mser.2005.04.002
Yan, W., Shen, N., Xiao, Y., Chen, Y., Sun, F., Kumar Tyagi, V., & Zhou, Y. (2017). The role of conductive materials in the start-up period of thermophilic anaerobic system. *Bioresource Technology*, *239*, 336–344. http://doi.org/10.1016/j.biortech.2017.05.046
Yang, Q., Luo, K., Li, X. ming, Wang, D. bo, Zheng, W., Zeng, G. ming, & Liu, J. jin. (2010). Enhanced efficiency of biological excess sludge hydrolysis under anaerobic digestion by additional enzymes. *Bioresource Technology*, *101*(9), 2924–2930. http://doi.org/10.1016/j.biortech.2009.11.012
Zhang, J., & Lu, Y. (2016). Conductive Fe3O4 nanoparticles accelerate syntrophic methane production from butyrate oxidation in two different lake sediments. *Frontiers in Microbiology*, *7*, 1–9. http://doi.org/10.3389/fmicb.2016.01316
Zhang, L., Petersen, E. J., Habteselassie, M. Y., Mao, L., & Huang, Q. (2013). Degradation of multiwall carbon nanotubes by bacteria. *Environmental Pollution*, *181*, 335–339. http://doi.org/10.1016/j.envpol.2013.05.058
Zhao, L., Ji, Y., Sun, P., Li, R., Xiang, F., Wang, H., ... Yang, Y. (2018). Effects of individual and complex ciprofloxacin, fullerene C60, and ZnO nanoparticles on sludge digestion: Methane production, metabolism, and microbial community. *Bioresource Technology*, *267*, 46–53. http://doi.org/10.1016/j.biortech.2018.07.024
Zhao, Z., Zhang, Y., Yu, Q., Dang, Y., Li, Y., & Quan, X. (2016). Communities stimulated with ethanol to perform direct interspecies electron transfer for syntrophic metabolism of propionate and butyrate. *Water Research*, *102*, 475–484. http://doi.org/10.1016/j.watres.2016.07.005
Ziganshin, A. M., Liebetrau, J., Pröter, J., & Kleinsteuber, S. (2013). Microbial community structure and dynamics during anaerobic digestion of various agricultural waste materials. *Applied Microbiology and Biotechnology*, *97*(11), 5161–5174. http://doi.org/10.1007/s00253-013-4867-0
Zinder, S. H. (1993). Phisiological Ecology of Methanogens. In *Methanogenesis: Ecology, Physiology,*

Biochemistry & Genetics, 1st ed. New York: Champman and Hall (p.130)

7. APPENDICES

Appendix I - Specific methanogenic activity tests

Specific methanogenic activity (SMA) of the anaerobic sludge was determined as detailed by Coates and collaborators (1996) and Colleran and collaborators (1992), following the method of the pressure transducer technique. The principle of the method consists in the monitorization of pressure variations in sealed vials, according to the biogas production and substrate degradation. For this purpose, a pressure transducer (Centrepoints Electronics, Galway, Ireland), that measures changes of ± 2 atm (0 ± 202.6 kPa) in the interval ranging from-200 to +200 mV, was used. This methodology implies a loss of approximately 30 μL of biogas in each pressure measurement, which is a negligible fraction of the total biogas produced during the test. At the end of the assay, the methane content of the liquid substrates vials was quantified by gas chromatography (GC), comparing the peak areas of the test vials with the one of the standard (40 % of CH_4). The GC analysis were performed in a GC Chrompack 9000, equipped with a Propack Q, 80/100 mesh column, with N_2/Air and Argon as carrier gases, at a flow of 30 and 5 mL/min, respectively. Injector, column and detector temperatures were 110, 35 and 220°C, respectively.

In order to convert the pressure measurements to volume of biogas produced, the headspace volume of each bottle was determined, by measuring the pressure increase when 5 mL of air were injected in the vials. The volatile solids (VS) were measured intended to determine the exact biomass content in each vial.

Calculation of methanogenic acetoclastic and acetogenic activity

The SMA for liquid substrates (Equation 7.1) was obtained by dividing the percentage of methane present in the biogas produced per day (Equation 7.2), by the VS present in each vial, taking into account the calibration factor of the pressure transducer. The values were expressed in $mLCH_4$@STP/gVSS.day.

$$SMA = \frac{MPD \times CF}{VS} \quad \text{Equation 7.1}$$

Where,

MPD = Methane produced per day

CF = Calibration factor of the pressure transducer

VS = Volatile solids present in each vial

$$MPD = \frac{mV/h}{mV/mL} \times 24 \times MP \quad \text{Equation 7.2}$$

Where,

MP = Methane percentage produced

Calculation of methanogenic hydrogenotrophic activity

The SMA for gaseous substrates (Equation 7.3) is based in the pressure decrease related to the conversion of H_2/CO_2 to CH_4 that corresponds to the conversion of 5 moles of H_2 and CO_2 in one mole of CH_4.

$$SMA = \frac{mL_{CH_4}/_h \times 24 \times CF}{VS} \quad \text{Equation 7.3}$$

Where:

CF = Calibration factor of the pressure transducer

VS = Volatile solids present in each vial

The methane produced per hour is achieved considering the mentioned stoichiometry of the reaction, which allow the direct conversion of the pressure measured along the assay to mL of CH_4 (Equation 7.4)

$$mL_{CH_4} = \frac{P_n - P_0}{mV/_{mL} \times 4} \quad \text{Equation 7.4}$$

Where:

P_n = Pressure measured in a certain time

P_0 = Initial pressure

Appendix II - Preparation of stock solutions

Ciprofloxacin (CIP) was provided by the company Sigma-Aldrich Química (Sintra, Portugal), at a purity over 98 %. A stock solution of 1250 mg/L of CIP was made in deionised water, adding approximately 250 µL HCl (2 M) until total dissolution of CIP, which resulted in a final pH of 2.5. Solutions with final concentrations of 625, 125, 62.5, 12.5, 6.25 and 0.625 mg/L were made from the stock solutions, in order to get the following concentrations in the test vials: 0.05, 0.5, 1, 5, 10, 50 and 100 mg/L. These solutions were stored in the freezer at 4 °C. It should be considered that before proceeding to the toxicity assays the pH of the buffer was measured in order to verify that the addition of the stock solution of CIP to the sodium bicarbonate buffer did not changed it.

Diclofenac (DCF) sodium salt was provided by the company Sigma-Aldrich Química (Sintra, Portugal) at a purity over 98 %. A stock solution of DCF stock solutions with a concentration of 1250

mg/L, was made in deionised water. Solutions with final concentrations of 625, 125, 62.5 and 12.5 mg/L were made from the stock solution, in order to have the following concentrations in the test vials: 1, 5, 10, 50 and 100 mg/L. These solutions were stored in the freezer at 4 °C.

Ibuprofen (IBP) was provided by the company Sigma-Aldrich Química (Sintra, Portugal), at a purity over 98 %. IBP stock solutions, with a concentration of 1250 mg/L, were made in deionised with an organic solvent, necessary for IBP dissolution. Three organic solvents were tested: acetonitrile, methanol and ethanol. Solutions with final concentrations of 125, 62.5, 12.5, 6.25 and 0.625 mg/L were made from the stock of 1250 mg/L of IBP with acetonitrile, in order to have the following concentrations in the test reactors: 20, 10, 5, 1, 0.5 and 0.05 mg/L. From the stock solution of 1250 mg/L of IBP with methanol another 3 were made with final concentrations of 625, 125 and 62.5 mg/L, making the dilutions only in methanol, as the presence of water led to precipitation of IBP. These solutions were stored in the freezer at 4 °C. The concentrations of the pharmaceutical present in the tests vials and corresponding concentrations of organic solvent can be seen in Table 7.1.

Table 7.1 - Concentrations of the different solutions of IBP and EE2 present in the tests vials and corresponding concentrations of organic solvent.

Pharmaceutical	[Pharmaceutical] (mg/L)	[Organic solvent] (mg/L)
IBP in acetonitrile	0.05	11
	1	226
	10	2260
IBP in methanol	5	61364
	10	59368
	50	43402
	100	23443
IBP in ethanol	100	28404
EE2 in ethanol	1	62785
	5	61447
	10	59775
	50	46393
	100	29666

17α-Ethinylestradiol (EE2) was provided by the company Sigma-Aldrich Química (Sintra, Portugal), at a purity over 98 %. A stock solution at 1250 mg/L of EE2 was made in deionised water, followed by addition of ethanol until total hormone dissolution. From the stock solution another 4 were made, diluting the stock solution in ethanol, with final concentrations of 625, 125, 62.5 and 12.5 mg/L, in order to get the following concentrations in the test vials: 100, 50, 10, 5 and 1 mg/L. These solutions were stored in the freezer at 4 °C. The concentrations of the pharmaceutical present in the test vials, and corresponding concentrations of organic solvent, can be accessed in Table 7.1.

Appendix III - Statistical analysis of the toxicity of pharmaceuticals towards methanogenic communities

Statistical analysis were performed for comparisons between the effects in the SMA of the different concentrations of model pharmaceuticals. To identify which concentrations were significantly different, the Tukey post-test was performed with significance level of 5 % and its results are presented in Table 7.2 to Table 7.7.

Table 7.2 – Summary of the statistical results comparing the effects of the different concentrations of ciprofloxacin in the methanogenic activity in the presence of different substrates.

		Acetate		**H_2/CO_2**		**VFA**	
	Tukey's multiple comparisons test	Significant?	Summary	Significant?	Summary	Significant?	Summary
Ciprofloxacin (mg/L)	0 vs. 0.05	No	ns	No	ns	No	ns
	0 vs. 0.5	No	ns	No	ns	No	ns
	0 vs. 1	No	ns	No	ns	Yes	*
	0 vs. 5	No	ns	No	ns	Yes	**
	0 vs. 10	No	ns	No	ns	Yes	**
	0 vs. 50	Yes	***	No	ns	Yes	*
	0 vs. 100	Yes	****	No	ns	Yes	***
	0.05 vs. 0.5	No	ns	No	ns	No	ns
	0.05 vs. 1	No	ns	No	ns	No	ns
	0.05 vs. 5	No	ns	No	ns	No	ns
	0.05 vs. 10	No	ns	No	ns	No	ns
	0.05 vs. 50	Yes	***	No	ns	No	ns
	0.05 vs. 100	Yes	***	No	ns	Yes	*

Continuation

		Acetate		H_2/CO_2		VFA	
	Tukey's multiple comparisons test	Significant?	Summary	Significant?	Summary	Significant?	Summary
Ciprofloxacin (mg/L)	0.5 vs. 1	No	ns	No	ns	No	ns
	0.5 vs. 5	No	ns	No	ns	No	ns
	0.5 vs. 10	No	ns	No	ns	No	ns
	0.5 vs. 50	Yes	**	No	ns	No	ns
	0.5 vs. 100	Yes	***	No	ns	No	ns
	1 vs. 5	No	ns	No	ns	No	ns
	1 vs. 10	No	ns	No	ns	No	ns
	1 vs. 50	No	ns	No	ns	No	ns
	1 vs. 100	No	ns	No	ns	No	ns
	5 vs. 10	No	ns	No	ns	No	ns
	5 vs. 50	Yes	*	No	ns	No	ns
	5 vs. 100	Yes	*	No	ns	No	ns
	10 vs. 50	Yes	***	No	ns	No	ns
	10 vs. 100	Yes	***	No	ns	No	ns

****- Extremely significant, p value < 0.0001; ***- Extremely significant, p value 0.0001 to 0.001; **- Very significant, p value 0.001 to 0.01; * - Significant, p value 0.01 to 0.05; Ns - Not significant, p value ≥ 0.05.

Table 7.3 - Summary of the statistical results comparing the effects of the different concentrations of diclofenac in the methanogenic activity in the presence of different substrates.

		Acetate		H_2/CO_2		VFA	
	Tukey's multiple comparisons test	Significant?	Summary	Significant?	Summary	Significant?	Summary
Diclofenac (mg/L)	0 vs. 1	No	ns	No	ns	No	ns
	0 vs. 5	No	ns	No	ns	No	ns
	0 vs. 10	No	ns	No	ns	No	ns
	0 vs. 50	Yes	*	No	ns	Yes	*
	0 vs. 100	Yes	***	No	ns	Yes	***
	1 vs. 5	No	ns	No	ns	No	ns
	1 vs. 10	No	ns	No	ns	No	ns
	1 vs. 50	No	ns	No	ns	No	ns
	1 vs. 100	Yes	**	No	ns	Yes	**
	5 vs. 10	No	ns	No	ns	No	ns

Continuation

		Acetate		H_2/CO_2		VFA	
	Tukey's multiple comparisons test	Significant?	Summary	Significant?	Summary	Significant?	Summary
Diclofenac (mg/L)	5 vs. 50	No	ns	No	ns	No	ns
	5 vs. 100	Yes	**	No	ns	Yes	**
	10 vs. 50	No	ns	No	ns	No	ns
	10 vs. 100	No	ns	No	ns	No	ns
	50 vs. 100	No	ns	No	ns	No	ns

****- Extremely significant, p value < 0.0001; ***- Extremely significant, p value 0.0001 to 0.001; **- Very significant, p value 0.001 to 0.01; * - Significant, p value 0.01 to 0.05; Ns - Not significant, p value ≥ 0.05.

Table 7.4 - Summary of the statistical results, comparing the effects of the different concentrations of ibuprofen, dissolved in acetonitrile, and the effect of acetonitrile, in the methanogenic activity, in the presence of different substrates.

		Acetate		H_2/CO_2		VFA	
	Tukey's multiple comparisons test	Significant?	Summary	Significant?	Summary	Significant?	Summary
[Ibuprofen] and [*acetonitrile*] (mg/L)	0 vs. 0.05	Yes	*	No	ns	No	ns
	0 vs. 0.5	Yes	*	No	ns	No	ns
	0 vs. 1	No	ns	No	ns	No	ns
	0 vs. 5	No	ns	No	ns	No	ns
	0 vs. 10	No	ns	No	ns	No	ns
	0 vs. 20	Yes	***	No	ns	No	ns
	0 vs. *11*	No	ns	Yes	*	No	ns
	0 vs. *226*	Yes	*	No	ns	No	ns
	0 vs. *2260*	Yes	*	No	ns	No	ns
	0.05 vs. 0.5	No	ns	No	ns	No	ns
	0.05 vs. 1	No	ns	No	ns	No	ns
	0.05 vs. 5	No	ns	No	ns	No	ns
	0.05 vs. 10	No	ns	No	ns	No	ns
	0.05 vs. 20	No	ns	No	ns	No	ns
	0.05 vs. *11*	No	ns	No	ns	No	ns
	0.05 vs. *226*	No	ns	No	ns	No	ns
	0.05 vs. *2260*	No	ns	No	ns	No	ns
	0.5 vs. 1	No	ns	No	ns	No	ns
	0.5 vs. 5	No	ns	No	ns	No	ns

Continuation

		Acetate		H_2/CO_2		VFA	
	Tukey's multiple comparisons test	Significant?	Summary	Significant?	Summary	Significant?	Summary
[Ibuprofen] and [*acetonitrile*] (mg/L)	0.5 vs. 10	No	ns	No	ns	No	ns
	0.5 vs. 20	No	ns	No	ns	No	ns
	0.5 vs. *11*	No	ns	No	ns	No	ns
	0.5 vs. *226*	No	ns	No	ns	No	ns
	0.5 vs. *2260*	No	ns	No	ns	No	ns
	1 vs. 5	No	ns	No	ns	No	ns
	1 vs. 10	No	ns	No	ns	No	ns
	1 vs. 20	No	ns	No	ns	No	ns
	1 vs. *11*	No	ns	No	ns	No	ns
	1 vs. *226*	No	ns	No	ns	No	ns
	1 vs. *2260*	No	ns	No	ns	No	ns
	5 vs. 10	No	ns	No	ns	No	ns
	5 vs. 20	No	ns	No	ns	No	ns
	5 vs. *11*	No	ns	No	ns	No	ns
	5 vs. *226*	No	ns	No	ns	No	ns
	5 vs. *2260*	No	ns	No	ns	No	ns
	10 vs. 20	No	ns	No	ns	No	ns
	10 vs. *11*	No	ns	No	ns	No	ns
	10 vs. *226*	No	ns	No	ns	No	ns
	10 vs. *2260*	No	ns	No	ns	No	ns
	20 vs. *11*	No	ns	Yes	*	No	ns
	20 vs. *226*	No	ns	No	ns	No	ns
	20 vs. *2260*	No	ns	Yes	*	No	ns
	11 vs. *226*	No	ns	No	ns	No	ns
	11 vs. *2260*	No	ns	No	ns	No	ns
	226 vs. *2260*	No	ns	No	ns	No	ns

****- Extremely significant, p value < 0.0001; ***- Extremely significant, p value 0.0001 to 0.001; **- Very significant, p value 0.001 to 0.01; * - Significant, p value 0.01 to 0.05; Ns - Not significant, p value ≥ 0.05.

Table 7.5 - Summary of the statistical results, comparing the effects of the different concentrations of ibuprofen, dissolved in methanol, and the effect of methanol, in the methanogenic activity in the presence of different substrates.

		Acetate		**H_2/CO_2**		**VFA**	
	Tukey's multiple comparisons test	Significant?	Summary	Significant?	Summary	Significant?	Summary
[Ibuprofen] and [*Methanol*] (mg/L)	0 vs. 5	No	ns	Yes	****	Yes	****
	0 vs. 10	Yes	**	Yes	****	Yes	****
	0 vs. 50	Yes	****	Yes	****	Yes	****
	0 vs. 100	No	ns	Yes	****	Yes	****
	0 vs. *61364*	No	ns	Yes	****	Yes	****
	0 vs. *59368*	Yes	**	Yes	****	Yes	****
	0 vs. *43402*	No	ns	Yes	****	Yes	****
	0 vs. *23443*	No	ns	No	ns	Yes	***
	5 vs. 10	No	ns	Yes	*	No	ns
	5 vs. 50	No	ns	Yes	*	No	ns
	5 vs. 100	No	ns	No	ns	No	ns
	5 vs. *61364*	No	ns	No	ns	No	ns
	5 vs. *59368*	No	ns	No	ns	No	ns
	5 vs. *43402*	No	ns	No	ns	No	ns
	5 vs. *23443*	No	ns	Yes	***	No	ns
	10 vs. 50	No	ns	No	ns	No	ns
	10 vs. 100	No	ns	Yes	*	No	ns
	10 vs. *61364*	No	ns	No	ns	No	ns
	10 vs. *59368*	No	ns	No	ns	No	ns
	10 vs. *43402*	No	ns	No	ns	No	ns
	10 vs. *23443*	Yes	*	Yes	****	No	ns
	50 vs. 100	Yes	*	No	ns	No	ns
	50 vs. *61364*	No	ns	No	ns	No	ns
	50 vs. *59368*	No	ns	No	ns	No	ns
	50 vs. *43402*	No	ns	No	ns	No	ns
	50 vs. *23443*	Yes	***	Yes	****	No	ns
	100 vs. *61364*	No	ns	No	ns	No	ns
	100 vs. *59368*	No	ns	No	ns	No	ns
	100 vs. *43402*	No	ns	No	ns	No	ns

Continuation

		Acetate		H_2/CO_2		VFA	
	Tukey's multiple comparisons test	Significant?	Summary	Significant?	Summary	Significant?	Summary
[Ibuprofen] and [*Methanol*] (mg/L)	100 vs. *23443*	No	ns	Yes	***	No	ns
	61364 vs. *59368*	No	ns	No	ns	No	ns
	61364 vs. *43402*	No	ns	No	ns	No	ns
	61364 vs. *23443*	No	ns	Yes	**	No	ns
	59368 vs. *43402*	No	ns	No	ns	No	ns
	59368 vs. *23443*	Yes	*	Yes	****	No	ns
	43402 vs. *23443*	No	ns	Yes	****	No	ns

****- Extremely significant, p value < 0.0001; ***- Extremely significant, p value 0.0001 to 0.001; **- Very significant, p value 0.001 to 0.01; * - Significant, p value 0.01 to 0.05; Ns - Not significant, p value ≥ 0.05.

Table 7.6 - Summary of the statistical results, comparing the effects of the 100 mg/L of ibuprofen, dissolved in ethanol, and the effect of ethanol, in the methanogenic activity, in the presence of different substrates.

		Acetate		H_2/CO_2		VFA	
	Tukey's multiple comparisons test	Significant?	Summary	Significant?	Summary	Significant?	Summary
[Ibuprofen] and [*Ethanol*] (mg/L)	0 vs. 100	Yes	***	No	ns	No	ns
	0 vs. *28404*	Yes	****	No	ns	No	ns
	100 vs. *28404*	Yes	**	No	ns	No	ns

****- Extremely significant, p value < 0.0001; ***- Extremely significant, p value 0.0001 to 0.001; **- Very significant, p value 0.001 to 0.01; * - Significant, p value 0.01 to 0.05; Ns - Not significant, p value ≥ 0.05.

Table 7.7 - Summary of the statistical results, comparing the effects of the different concentrations of 17α-ethinylestradiol, dissolved in ethanol, and the effect of ethanol, in the methanogenic activity in the presence of different substrates.

		Acetate		H_2/CO_2		VFA	
	Tukey's multiple comparisons test	Significant?	Summary	Significant?	Summary	Significant?	Summary
[17α-ethinylestradiol] and [*Ethanol*] (mg/L)	0 vs. 1	Yes	****	Yes	****	Yes	***
	0 vs. 5	Yes	****	Yes	****	Yes	**
	0 vs. 10	Yes	****	Yes	***	Yes	***
	0 vs. 50	Yes	****	Yes	****	No	ns
	0 vs. 100	Yes	**	Yes	****	No	ns

Continuation

		Acetate		H_2/CO_2		VFA	
	Tukey's multiple comparisons test	Significant?	Summary	Significant?	Summary	Significant?	Summary
[17α-ethinylestradiol] and [*Ethanol*] (mg/L)	0 vs. *62785*	Yes	***	Yes	****	Yes	***
	0 vs. *61447*	Yes	****	Yes	****	Yes	***
	0 vs. *59775*	Yes	****	Yes	***	No	ns
	0 vs. *46393*	No	ns	Yes	***	No	ns
	0 vs. *29666*	Yes	***	Yes	****	No	ns
	1 vs. 5	No	ns	No	ns	No	ns
	1 vs. 10	No	ns	No	ns	No	ns
	1 vs. 50	No	ns	No	ns	No	ns
	1 vs. 100	No	ns	No	ns	Yes	****
	1 vs. *62785*	No	ns	No	ns	No	ns
	1 vs. *61447*	No	ns	No	ns	No	ns
	1 vs. *59775*	No	ns	No	ns	No	ns
	1 vs. *46393*	Yes	**	No	ns	Yes	**
	1 vs. *29666*	Yes	****	No	ns	Yes	****
	5 vs. 10	No	ns	No	ns	No	ns
	5 vs. 50	No	ns	No	ns	No	ns
	5 vs. 100	No	ns	No	ns	Yes	****
	5 vs. *62785*	No	ns	No	ns	No	ns
	5 vs. *61447*	No	ns	No	ns	No	ns
	5 vs. *59775*	No	ns	No	ns	No	ns
	5 vs. *46393*	Yes	**	No	ns	Yes	**
	5 vs. *29666*	Yes	****	No	ns	Yes	****
	10 vs. 50	No	ns	No	ns	No	ns
	10 vs. 100	Yes	**	No	ns	Yes	****
	10 vs. *62785*	No	ns	No	ns	No	ns
	10 vs. *61447*	No	ns	No	ns	No	ns
	10 vs. *59775*	No	ns	No	ns	No	ns
	10 vs. *46393*	Yes	****	No	ns	Yes	***
	10 vs. *29666*	Yes	****	No	ns	Yes	****
	50 vs. 100	No	ns	No	ns	Yes	**

Continuation

		Acetate		H_2/CO_2		VFA	
	Tukey's multiple comparisons test	Significant?	Summary	Significant?	Summary	Significant?	Summary
[17α-ethinylestradiol] and [*Ethanol*] (mg/L)	50 vs. *62785*	No	ns	No	ns	No	ns
	50 vs. *61447*	No	ns	No	ns	No	ns
	50 vs. *59775*	No	ns	No	ns	No	ns
	50 vs. *46393*	Yes	**	No	ns	No	ns
	50 vs. *29666*	Yes	****	No	ns	Yes	**
	100 vs. *62785*	No	ns	No	ns	Yes	****
	100 vs. *61447*	No	ns	No	ns	Yes	****
	100 vs. *59775*	No	ns	No	ns	Yes	**
	100 vs. *46393*	No	ns	No	ns	No	ns
	100 vs. *29666*	Yes	****	No	ns	No	ns
	62785 vs. *61447*	No	ns	No	ns	No	ns
	62785 vs. *59775*	No	ns	No	ns	No	ns
	62785 vs. *46393*	Yes	**	No	ns	Yes	***
	62785 vs. *29666*	Yes	****	No	ns	Yes	****
	61447 vs. *59775*	No	ns	No	ns	No	ns
	61447 vs. *46393*	Yes	**	No	ns	Yes	***
	61447 vs. *29666*	Yes	****	No	ns	Yes	****
	59775 vs. *46393*	Yes	**	No	ns	No	ns
	59775 vs. *29666*	Yes	****	No	ns	Yes	**
	46393 vs. *29666*	Yes	***	No	ns	No	ns

****- Extremely significant, p value < 0.0001; ***- Extremely significant, p value 0.0001 to 0.001; **- Very significant, p value 0.001 to 0.01; * - Significant, p value 0.01 to 0.05; Ns - Not significant, p value ≥ 0.05.

Appendix IV – Effect of ibuprofen from stock solutions prepared with acetonitrile, methanol or ethanol, and of the solvents towards methanogenic communities

To evaluate the toxicity of IBP towards the methanogenic community, incubations with IBP dissolved in several solvents were performed: acetonitrile, methanol and ethanol and variations in SMA and respective inhibitions were analysed (Table 7.8).

Table 7.8 - Specific methanogenic activity ($mLCH_4$@SPT/gVSS.day) and percentage of SMA inhibition (I) in the presence of different substrates, at increasing concentrations of ibuprofen, prepared from stock solutions containing acetonitrile, methanol or ethanol. The SMA and inhibition of control, for the effect of organic solvent with substrate (OSS) and without substrate (OS), is also presented

Concentration		Substrate					
		Acetate		H_2/CO_2		VFA mixture	
		SMA ($mLCH_4$@SPT/gVSS.day)	I (%)	SMA ($mLCH_4$@SPT/gVSS.day)	I (%)	SMA ($mLCH_4$@SPT/gVSS.day)	I (%)
IBP (mg/L)	0	92 ± 7	0	539 ± 8	0	91 ± 3	0
	0.05	65 ± 3	30 ± 6	545 ± 27	0	81 ± 2	10 ± 4
	0.5	68 ± 8	26 ± 10	550 ± 20	0	82 ± 3	10 ± 5
	1	69 ± 3	24 ± 6	563 ± 29	0	82 ± 2	10 ± 3
	5	72 ± 12	22 ± 14	596 ± 7	0	88± 7	2 ± 8
	10	69 ± 5	25 ± 8	568 ± 19	0	80± 7	11 ± 9
	20	50 ± 6	45 ± 8	536 ± 56	0	81 ± 7	11 ± 9
ACN (mg/L)	11	67 ± 4	27 ± 7	644 ± 7	0	86 ± 11	0
	226	63 ± 3	32 ± 6	609 ± 8	0	76 ± 21	0
	2260	63 ± 2	31 ± 6	537 ± 8	0	83 ± 4	9 ± 5
IBP (mg/L)	0	97 ± 3	0	477 ± 8	0	138 ± 6	0
	5	79 ± 4	19 ± 5	366 ± 7	23 ± 2	88 ± 2	36 ± 3
	10	73 ± 3	25 ± 4	314 ± 21	34 ± 5	79 ± 8	43 ± 7
	50	62 ± 4	36 ± 4	315 ± 18	34 ± 4	78 ± 8	43 ± 6
	100	84 ± 2	13 ± 3	360 ± 3	24 ± 2	87 ± 3	37 ± 4
Methanol[a] (M)	1.92	80 ± 1	17 ± 3	365 ± 18	24 ± 4	87 ± 7	37 ± 6
	1.85	69 ± 2	29 ± 3	339 ± 16	29 ± 4	76 ± 4	45 ± 4
	1.35	78 ± 5	20 ± 6	336 ± 8	30 ± 2	76 ± 5	45 ± 4
	0.73	96 ± 6	0	447 ± 15	6 ± 3	98 ± 1	29 ± 3
		Methanol					
Methanol[b] (M)	1.92	75 ± 5					
	1.85	68 ± 7					
	1.35	98 ± 7					
	0.73	101 ± 10					

Continuation

Concentration		Acetate		H_2/CO_2		VFA mixture	
		SMA ($mLCH_4$@SPT/ gVSS.day)	I (%)	SMA ($mLCH_4$@SPT/ gVSS.day)	I (%)	SMA ($mLCH_4$@SPT/ gVSS.day	I (%)
IBP (mg/L)	0	87 ± 3	0	406 ± 36	0	84 ± 6	0
	100	111 ± 1	0	374 ± 1	0	109 ± 19	0
Ethanol[a] (M)	0.62	127 ± 4	0	376 ± 3	0	119± 1	0
		Ethanol					
Ethanol[b] (M)	0.62	189 ± 13					

a – in the presence of substrate; b - without substrate

Values with higher standard deviation than the median values and negative values the percentage of inhibition was considered zero.

The acetonitrile concentrations 2260, 226 and 11 mg/L correspond to the acetonitrile concentrations present in the assays performed with 0.05, 1 and 10 mg/L IBP, respectively; The methanol concentrations 1.92, 1.85, 1.35 and 0.73 M correspond to the methanol concentrations present in the assays performed with 5, 10, 50 and 100 mg/L IBP, respectively; The ethanol concentration 0.62 M correspond to the ethanol concentration present in the assay with 100 mg/L IBP.

Appendix V – Methanogenic activity in ethanol

To evaluate if the ethanol was used as substrate by the anaerobic sludge, and if and how much the SMA change with the increasing concentrations of ethanol, present at the assays with EE2, incubations with ethanol and without the addition of the others substrates tested, were performed, and variations in SMA were analysed (Table 7.9)

Table 7.9 - Specific methanogenic activity ($mLCH_4$@SPT/gVSS.day) in the presence of different concentrations of ethanol and without substrate (OS).

Ethanol (M)	SMA ($mLCH_4$@SPT/gVSS.day)
1.36	97 ± 6
1.33	92 ± 1
1.30	91 ± 3
1.00	117 ± 6
0.64	156 ± 3

Appendix VI - UHPLC and HPLC calibration curves

In order to make a correspondence between the areas obtained by UHPLC analysis of pharmaceuticals and HPLC analysis of VFA, calibration curves were made for all the compounds: DCF (Figure 7.1), IBP (Figure 7.2), EE2 (Figure 7.3), acetate, butyrate, propionate and ethanol (Figure 7.4).

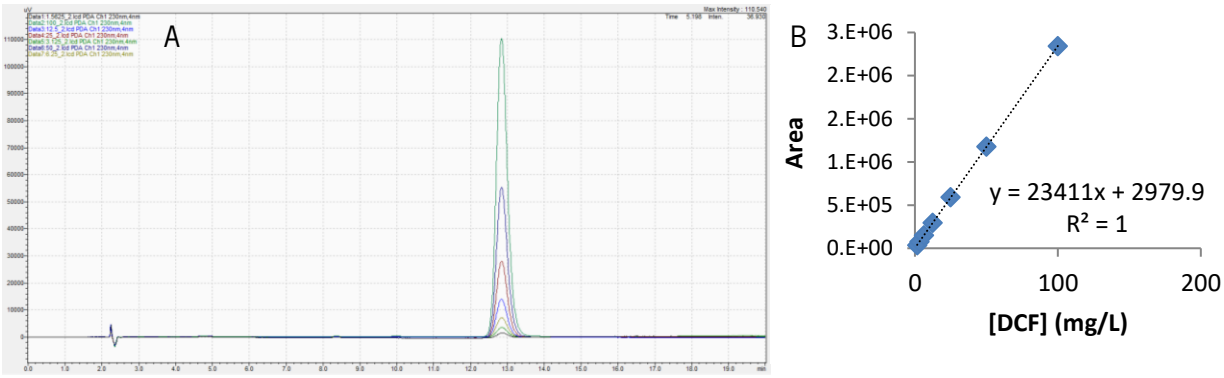


Figure 7.1 – UHPLC Chromatograms (A) and calibration curve (B), of DCF at the concentrations 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 mg/L.

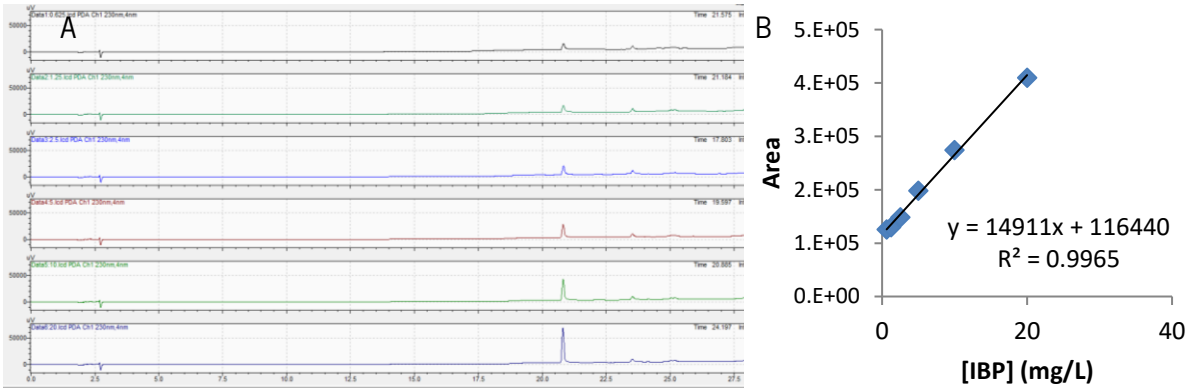


Figure 7.2 – UHPLC Chromatograms (A) and calibration curve (B), of IBP in the concentrations 0.625, 1.25, 2.4, 5, 10 and 20 mg/L.

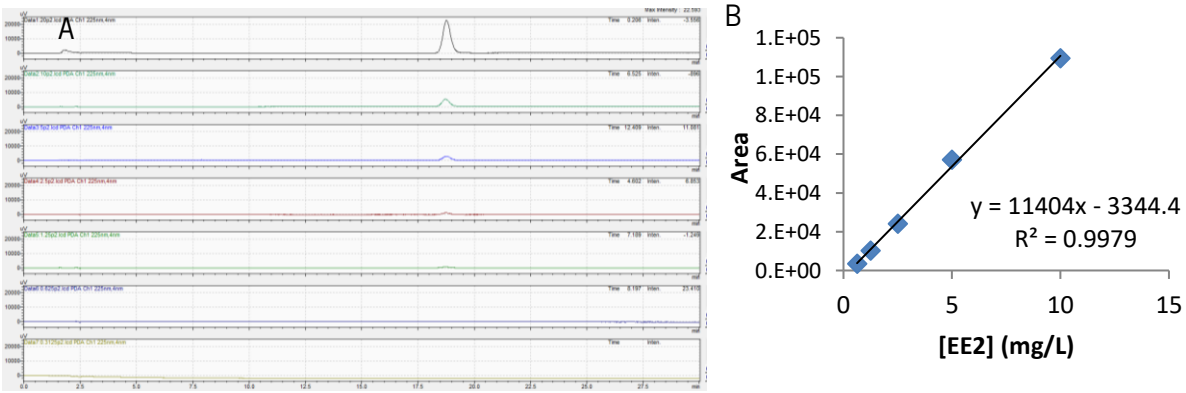


Figure 7.3– UHPLC Chromatograms (A) and calibration curve (B), of EE2 in the concentrations 20, 10, 5, 2.5, 1.25, 0.625 and 0.313 mg/L.

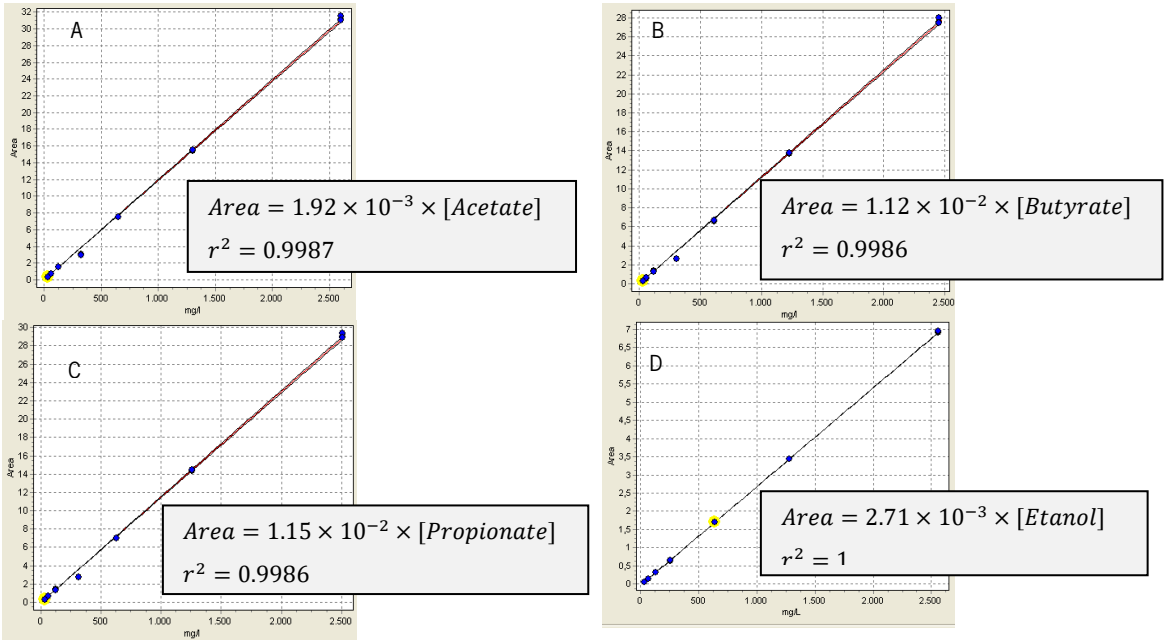


Figure 7.4 – HPLC calibration curve of (A) - Acetate; (B) -Butyrate ; (C) - Propionate and (D) – Ethanol in the concentrations of 30, 60, 120, 315, 630, 1250 and 2500 mg/L

Appendix VII - Chromatograms of biodegradation

Chromatographs for 0 and 48 h of reaction in the biologic assay with DCF, CNT and VFA and respective abiotic control are presented in Figure 7.5.

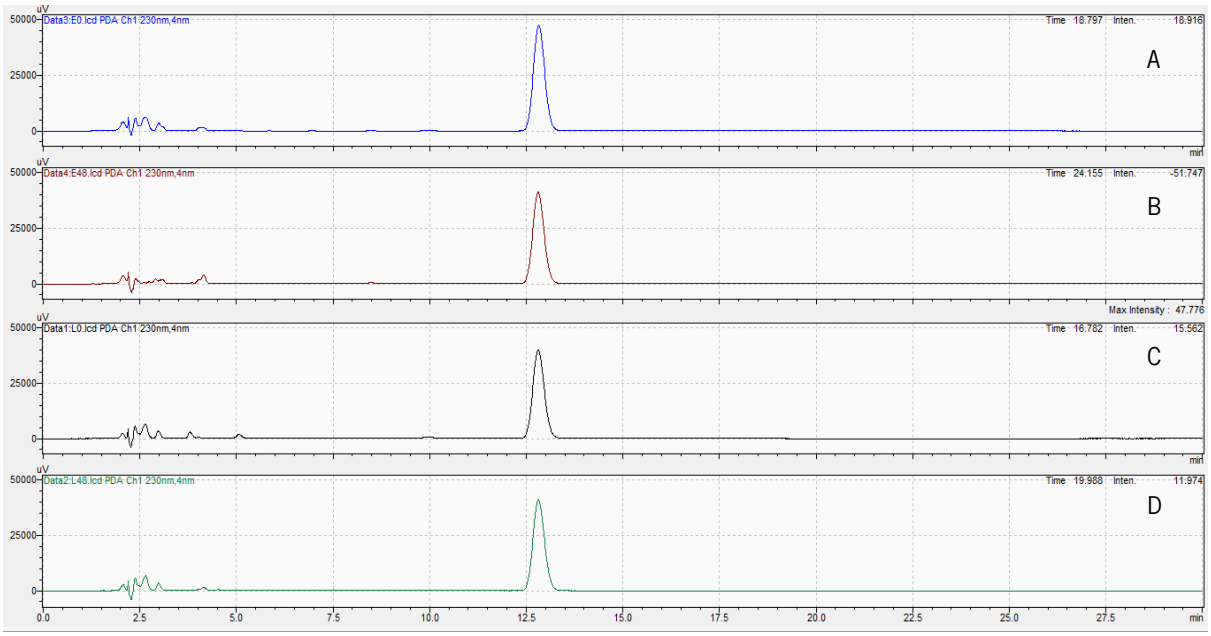


Figure 7.5 - UHPLC chromatograms at 230 nm of the diclofenac removal assays with CNT and VFA: (A) Biological assay at 0 h reaction; (B) Biological assay at 48 h reaction; (C) Abiotic control at 0 h of reaction and (D) Abiotic control at 48 h of reaction.

Chromatographs for 0 and 48 h of reaction in the biologic assay with IBP, CNT and VFA and respective abiotic control are presented in Figure 7.6.

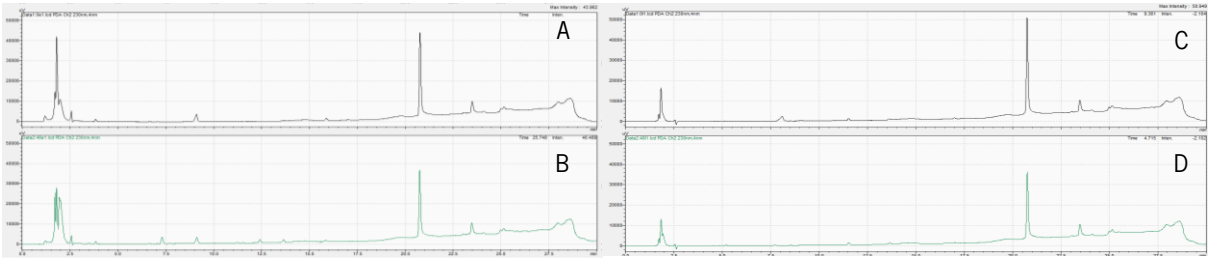


Figure 7.6 - UHPLC chromatograms at 230 nm of the ibuprofen removal assays with CNT and VFA: (A) Biological assay at 0 h reaction; (B) Biological assay at 48 h reaction; (C) Abiotic control at 0 h of reaction and (D) Abiotic control at 48 h of reaction.

Chromatographs for 0 and 48 h of reaction in the biologic assay with EE2 and VFA and EE2, CNT and VFA and respective abiotic controls are presented in Figure 7.7

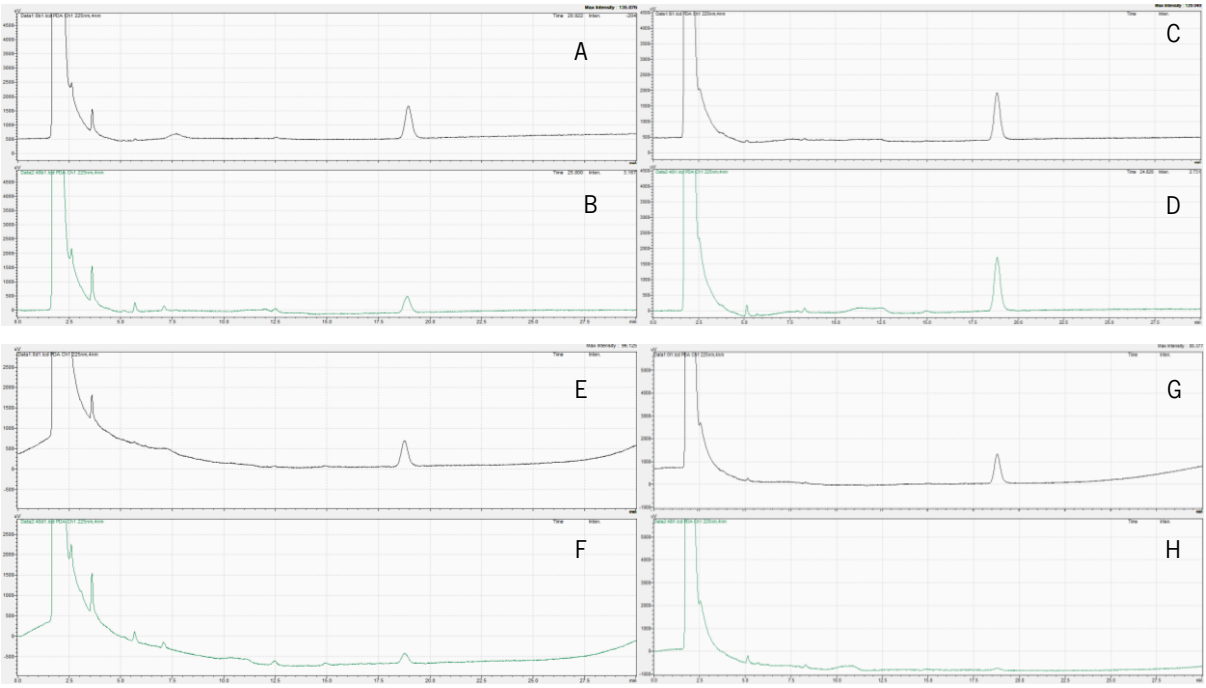


Figure 7.7 - UHPLC chromatograms at 225 nm of the 17α-ethinylestradiol removal assays with VFA: (A) - Biological assay at 0 h reaction; (B) - Biological assay at 48 h reaction; (C) - Abiotic control at 0 h of reaction; (D) - Abiotic control at 48 h of reaction; and 17α-ethinylestradiol removal assays with CNT and VFA: (E) - Biological assay at 0 h reaction; (F) - Biological assay at 48 h reaction; (G) - Abiotic control at 0 h of reaction and (H) - Abiotic control at 48 h of reaction