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Magnetic carbon nanotubes as a new generation of electron shuttles for anaerobic removal of pharmaceuticals

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RESUMO

Os fármacos e os seus metabolitos são considerados poluentes emergentes devido à sua entrada continua no ambiente aquático, representando, portanto, um problema de saúde pública. Os sistemas de tratamento convencionais não são eficazes na remoção deste tipo de compostos, os quais surgem muitas vezes concentrados na linha de lamas, frequentemente estabilizada por digestão anaeróbia. No entanto, a taxa de redução anaeróbia é lenta devido a limitações associadas à transferência de eletrões, podendo ser aceleradas através de mediadores redox (RM). Os materiais de carbono (CM) têm sido utilizados como RM viáveis. Este trabalho focou-se no efeito dos nanotubos de carbono (CNT) e dos nanotubos de carbono impregnados com 2 % de Ferro (CNT@2%Fe) como RM na redução anaeróbia da Ciprofloxacina (CIP).

Nos ensaios biológicos na ausência de CM, a remoção de CIP após 24 h de reação foi de 72 % e na presença de CM, a remoção de CIP foi de 98 % nos ensaios com CNT e de 92 % nos ensaios com CNT@2%Fe. Através dos ensaios de toxicidade, verificou-se que os CM não inibem a atividade biológica nos diferentes substratos, enquanto a CIP inibe cerca de 45 % na presença de etanol. Foram ainda realizados testes com a bactéria *Vibrio fischeri* para avaliar o efeito tóxico dos CM, da CIP e das soluções de CIP após 24 h de tratamento. Os resultados obtidos demonstraram que os CNT@2%Fe são mais tóxicos (35 %) do que os CNT (28 %) para a bactéria em questão. A toxicidade da CIP foi de 56 %, no entanto, as soluções tratadas na presença dos CM inferem relativamente baixa toxicidade, nomeadamente, 27 % na presença de CNT e 35 % na presença CNT@2%Fe, valores próximos dos obtidos com os materiais, pelo que, relativamente à CIP, há uma destoxificação, tornando viável o processo proposto.

Palavras-Chave: Ciprofloxacina; Compostos farmacêuticos; Mediadores Redox; Nanomateriais; Redução anaeróbia.

ABSTRACT

Pharmaceuticals and their metabolites are considered emerging pollutants due to their continuous input into the aquatic environment, representing a public health problem. The conventional treatment systems are not effective in the removal of these type of compounds, which are usually concentrated in the sludge line that is often stabilized by anaerobic digestion. However, the anaerobic reduction rate is slow due to limitations associated with electron transfer, which might be accelerated through redox mediators (RM). Carbon materials (CM) have been used as viable RM. This work focused on the effect of carbon nanotubes (CNT) and carbon nanotubes impregnated with 2 % of Iron (CNT@2%Fe) as RM for the anaerobic reduction of Ciprofloxacin (CIP).

In biological assays, in the absence of CM, the removal of CIP after 24 h reaction was 72 % and in the presence of the CM the CIP removal was 98 % in the assays with CNT and 92 % in the assays with CNT@2%Fe. Through toxicity assays, it was verified that CM did not inhibit the biological activity in different substrates, while the CIP inhibits around 45 % in the presence of the ethanol. Furthermore, tests have been made with the bacterium *Vibrio fischeri* to evaluate the toxic effect of the CM, CIP and the CIP solutions after 24 h of treatment. The results obtained demonstrated that for these bacteria CNT@2%Fe are more toxic than CNT. CIP toxicity was 56 %. Nevertheless, treated solutions in the presence of CM inferred low toxicity, namely, 27 % in the presence of CNT and 35 % in the presence of CNT@2%Fe, meaning detoxification and validating the proposed process.

KEYWORDS: Ciprofloxacin; Pharmaceuticals; Redox mediators; Nanomaterials; Anaerobic Reduction.

INDEX

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS INDEX

ACN	Acetonitrile
AOP	Advanced Oxidation Processes
CH_4	Methane
CM	Carbon Materials
C@MNP	Magnetic carbon composites
CNT	Carbon Nanotubes
CNT@2%Fe	Carbon Nanotubes impregnated with 2 % of Fe
CO_2	Carbon Dioxide
CIP	Ciprofloxacin
Fe	Iron
FID	Flame Ionization Detector
GC	Gas Chromatography
H_2	Hydrogen
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
INH	Luminescence Inhibition
IBP	Ibuprofen
MWCNT	Multiple Wall CNT
MNP	Magnetic Nanoparticles
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NH_4	Ammonium
NH_3	Ammonia
OH	Hydroxyl radicals
OD	Optical Density
PPCP	Pharmaceuticals and Personal Care Products
PR	Pharmaceutical Removal
RM	Redox Mediators
Rt	Retention Time
TS	Total Solids
VS	Volatile Solids
WWTP	Wastewater Treatment Plants

1. INTRODUCTION

Water is essential for the survival of all living creatures (Rahman et al., 2009). The exponential population growth, the ongoing climate changes, the scientific and technological advances of modern life, as well as the poor water management, with the aggravation of the excessive water resources usage by the different industries, contribute to water scarcity and to its increasing contamination (Bouwer, 2000; Jhansi and Mishra, 2013; Rahman et al., 2009). Keeping this in mind, assuring the water quality must be seen as a challenge for humanity in the XXI century (Schwarzenbach et al., 2010), with the focus on preserving, decontaminating and reusing water (Jhansi and Mishra, 2013).

According to the law *Decreto Lei no 152/97 de 19 de Junho* (1997), wastewaters result from the use of water in different anthropogenic activities (domestic, agricultural, industrial, and medical; among others) that alter the water quality. Nowadays, wastewaters are submitted to specific treatments prior reuse or discharge (Jhansi and Mishra, 2013). After treatment, the treated water has been used for agricultural and landscape irrigation, groundwater recharges, industrial applications, dual-distribution systems for flushing toilets and other urban uses (Angelakis and Snyder, 2015; Jhansi and Mishra, 2013; Le-Minh et al., 2010). The benefit of reusing these waters are the reduction of discharged effluents into nature and the spare of the existing water resources (Watkinson et al., 2007).

Over the past years, there has been a growing awareness about the presence in residual waters of polluting components known as emerging pollutants (Bouwer, 2000). According to several authors (Farré et al., 2008; Verlicchi et al., 2010), many of these compounds are still not contemplated in the water quality regulation, as they have not been fully studied previously, and, consequently, they represent a threat to the environment and public health. The principal emerging pollutants are fertilisers and pesticides used in agriculture (Bouwer, 2000), steroids and hormones (Farré et al., 2008), surfactants (Farré et al., 2008), solvents (Deblonde et al., 2011), dyes (Deblonde et al., 2011), heavy metals (Deblonde et al., 2011), endocrine disrupters (Farré et al., 2008), illicit drugs (Farré et al., 2008) and petrol additives (Farré et al., 2008). More recently, pharmaceuticals and personal care products (PPCP) have also been acknowledged as emerging contaminants, given their persistent presence in aquatic environments (Carlsson et al., 2006).

1.1 Environmental and health impact of pharmaceuticals

In the last years, there has been a substantial increase on the consumption of pharmaceutical compounds (Sangion and Gramatica, 2016). The potential impact on the environment, especially on the aquatic environment, has been investigated and has turned into an emerging research area.

For instance, Pereira et al. (2016) have identified the presence of pharmaceutical compounds such as anxiolytics, lipid regulators, antibiotics and anti-inflammatories both in the influent and in the effluent lines of 15 Wastewater Treatment Plants (WWTP), in 5 different regions of Portugal. Antibiotics and anti-inflammatories were the main compounds found, with average concentrations of 3300 and 15600 ng/L in the influents, respectively. In the effluents, these two types of compounds were present at average values of 900 and 1800 ng/L, respectively.

The direct exposure of humans to residual pharmaceuticals, either by ingestion of potable water or contaminated food which was washed or irrigated with contaminated water, is also a public health preoccupation (Jones et al., 2005; Schwarzenbach et al., 2010). Even though the amount of pharmaceuticals in drinking water is considered to represent minimal quantities,- nanograms (ng) up to micrograms (µg) per litre, the continuous input can instigate, at long term, major negative effects on all living organisms (Carvalho and Santos, 2016; Oller et al., 2011; Sangion and Gramatica, 2016). However, the health impact is not consensual. As example, Kümmerer (2009c) considered the risk for human beings by the ingestion of pharmaceutical compounds through the consumption of potable water as insignificant, but many authors, namely Luo et al., (2010) and Pruden et al., (2006), affirm that the use of pharmaceuticals, antibiotics more exactly, and their presence even at very small concentrations in wastewaters, can accelerate the development of antibiotic resistant genes. Furthermore, these compounds can disturb the microbial ecology, increasing the proliferation of pathogenic agents resistant to antibiotics, known as antibiotic resistant bacteria, leading to serious health risks for humans and animals (Fick et al., 2009; Xi et al., 2009; Zhang et al., 2009).

The resistant bacteria are capable of adapting themselves to changes, and to survive in unfavourable conditions (Kümmerer, 2009b). The *Staphylococcus aureus*, resistant to methicillin, and the *Enterococcus*, resistant to vancomycin, are two examples of found resistant bacteria (Kümmerer, 2009a). The transmission of these bacteria to humans can occur through water or food, *e.g.* if the plants are irrigated with superficial water or sewage; if manure is used as fertiliser or if there are resistant bacteria in the meat (Kümmerer, 2009a). Figure 1 represents this worldwide public health problem, schematizing the routes of antibiotics from anthropogenic activities to the environment and then to humans.

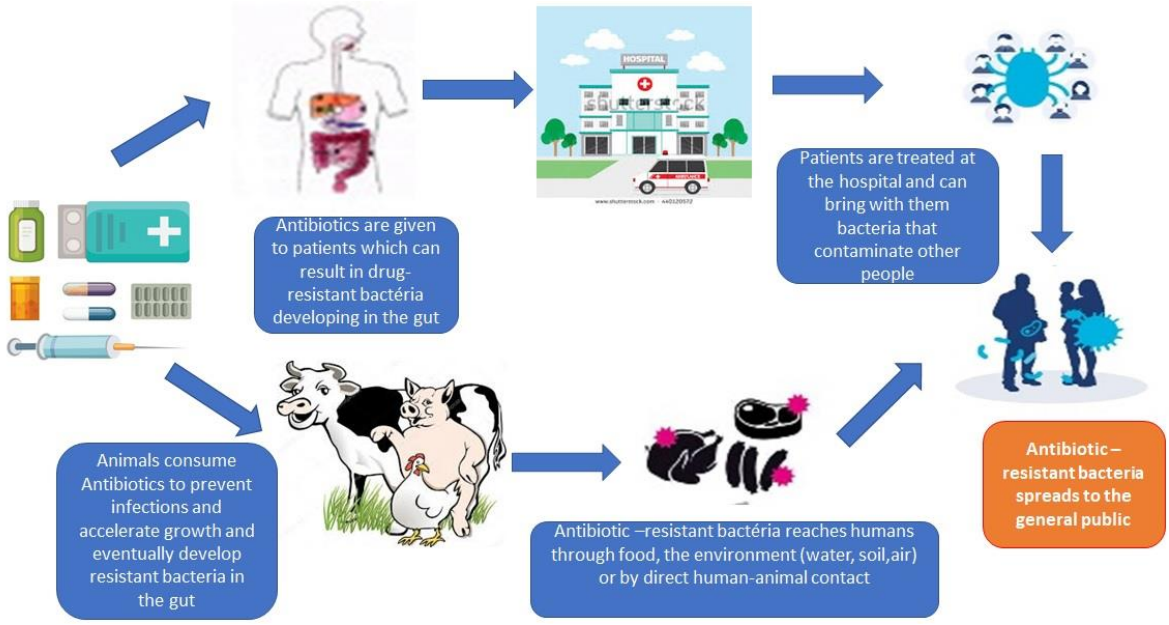

Figure 1. Antibiotic Resistance – how it spreads. Adapted from World Health Organization (2016).

1.2 The process of water contamination by pharmaceuticals

Pharmaceuticals present different functions and physicochemical and biological properties (Kümmerer, 2009b), which are chemically designed for preventive, diagnostical and therapeutic purposes (Kümmerer, 2009c). The pharmaceutical components are generally lipophiles, allowing them to pass through the biological membranes and to be more easily absorbed (Silva et al., 2015). They can be classified in different groups, in accordance with their effect or biological activity. Among them, are the classes of antibiotics, analgesics, betablockers, contraceptives, lipid regulators and antidepressants (Deblonde et al., 2011; Khetan and Collins, 2007; Kümmerer, 2009a). The antibiotics are classified according to their chemical behaviour as β-lactams, cephalosporin, penicillin, and quinolones (Kümmerer, 2009a).

They can be classified as persisting in the environment, given that they are resistant to the degradation processes existing in the natural environment (Sangion and Gramatica, 2016). Consequently, they are discharged and accumulated in the aquatic environment (Homem and Santos, 2011), contributing to the development and the global dissemination of antibiotic resistance (Carvalho and Santos, 2016; Kümmerer, 2009c; Sangion and Gramatica, 2016).

The ingestion of pharmaceuticals by humans and animals undergoes a partial structural alteration inside the organism, giving origin to metabolites and transformation products which are then excreted through urine or faeces (Carvalho and Santos, 2016; Khetan and Collins, 2007; Kümmerer, 2009a). The excretion of the pharmaceutical by these living creatures represent a potential direct source of contamination, affecting soils, groundwater resources and drinking waters directly through surface runoffs and leaching – (Figure 2) (Carvalho and Santos, 2016; Kemper, 2008; Klavarioti et al., 2009). Once released into the environment, some pharmaceuticals can suffer new alterations *(e.g.* biodegradation, chemical and photochemical degradation) that contribute to their elimination. Depending on their chemical structure, these compounds may undergo different transformations, giving rise to some products with different behaviour and ecotoxicological profile (Farré et al., 2008). The metabolism of pharmaceuticals by humans and animals can result in compounds with a higher toxic level than the original compounds (Kümmerer, 2009c).These by-products (metabolites and transformation products) together with the original pharmaceuticals are continuously released into the aquatic environments through municipal, agricultural and pharmaceutical industry sewages (Behera et al., 2011; Klavarioti et al., 2009). Among the antibiotics administrated to humans and animals, 30 % up to 90 % are excreted largely unmetabolized into the waste stream. These components cannot be removed efficiently by the conventional wastewater treatments (Gadipelly et al., 2014; Klavarioti et al., 2009; Kümmerer, 2009a).

Another source of pharmaceuticals into the environment is the improper disposal of expired medicines, often eliminated through domestic sewage, contributing to the contamination of superficial and underground water (Kümmerer, 2009a) – (Figure 2). As shown by a recently held study by Valormed in Portugal: "On the total amount of packages and pharmaceuticals sold last year, only 12 % have been recycled through the system for empty packages and expired pharmaceuticals" (RTP, 2017).

So, when it comes to the water pollution through pharmaceutical products, the WWTP are to be considered as the main source, due to the fact that they receive the components continuously and are not, however, able to degrade them readily (Gadipelly et al., 2014; Kümmerer, 2009c; Schwarzenbach et al., 2010). One of the difficulties for the removal of pharmaceuticals in the WWTP is their low concentrations in wastewater streams (Gartiser et al., 2007; Verlicchi et al., 2010).

The hospitals and the pharmaceutical industry represent also a great source of water contamination due to the significant liberation of micropollutants resulting from diagnostic, laboratory and investigation activities (Homem and Santos, 2011; Sangion and Gramatica, 2016; Verlicchi et al., 2010).

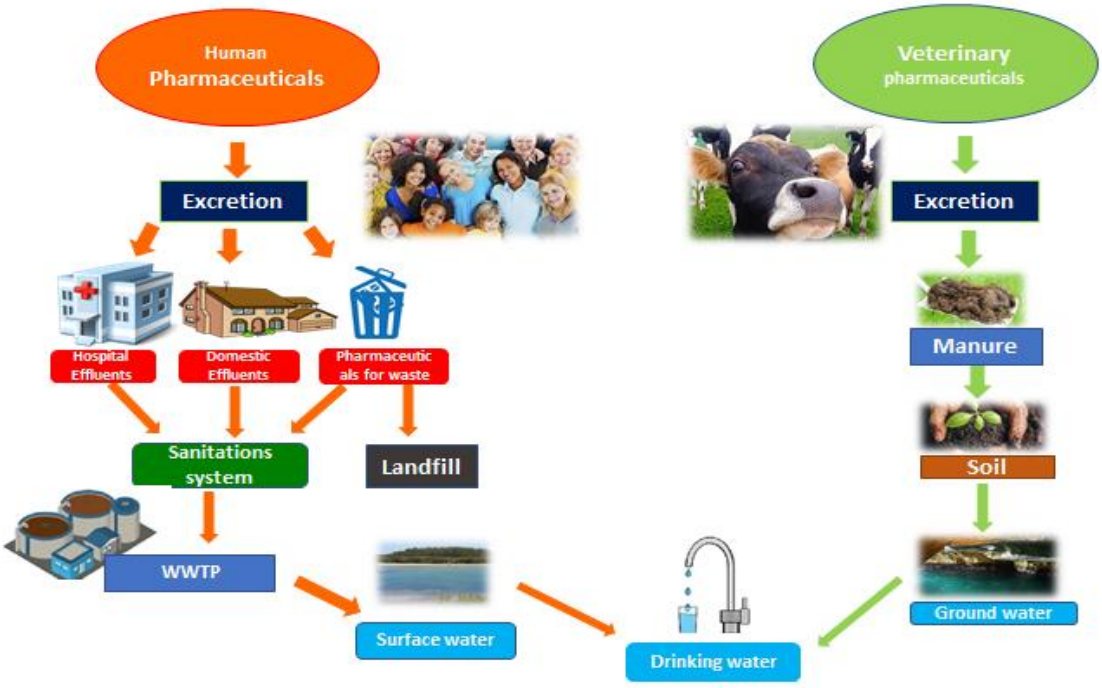

Figure 2. Fate of pharmaceuticals in the environment.

From the different types of pharmaceuticals used in Portugal, antibiotics, anti-inflammatory, and analgesics are the most prescribed and consumed (Pereira et al., 2015). Ciprofloxacin (CIP) (antibiotic) and ibuprofen (IBP) (non-steroidal anti-inflammatory) are two prime examples of the above mentioned. CIP (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid) – Figure 3, is one of the most used antibiotics in the entire world (Bojer et al., 2017; Espinosa-Mansilla et al., 2006; Sun et al., 2016). It belongs to the group of fluoroquinolones and it is used for a wide range of human or animal health problems, such as respiratory infections (Turiel et al., 2005), urinary tract infections (Turiel et al., 2005), skin infections (Bojer et al., 2017) and sexually transmitted diseases (Bojer et al., 2017). Due to its broad range of use, CIP has been detected in aquatic environment (Liao et al., 2016).

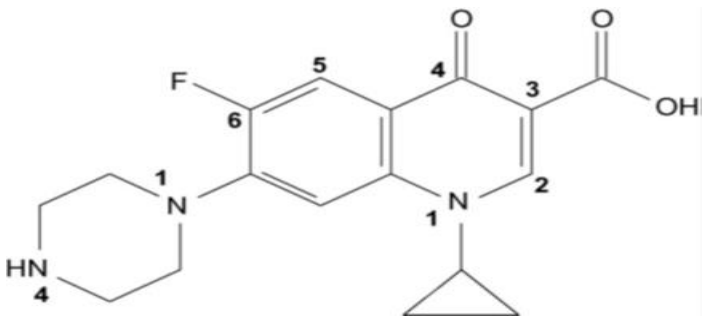

Figure 3. Chemical structure of CIP. Adapted from Vasconcelos et al. (2009).

IBP (2-(4-isobutylphenyl)propionic acid) – Figure 4, is a non-steroidal anti-inflammatory, which can be purchased over-the-counter in almost every country, and is broadly used to treat pain, fever, inflammations and also for musculoskeletal treatment (*e.g.* rheumatoid arthritis) (Espinosa-Mansilla et al., 2006; Langenhoff et al., 2013; Quero-Pastor et al., 2014).

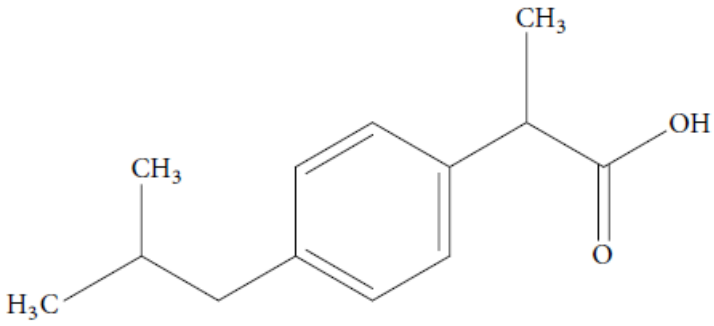

Figure 4. Chemical structure of IBP. Adapted from Langenhoff et al. (2013).

A large part of the administrated CIP by humans is excreted through urine (45 - 62 %) and faeces (15 - 25 %), without being metabolised (Girardi et al., 2011), reaching the aquatic environment through different ways: WWTP releases; leaching of sanitary landfills; discharges directly from pharmaceutical industries into the water; among others (Girardi et al., 2011). In relation to IBP, only 15 % are excreted unaltered through urine, whilst 2-hydroxy IBP (2-OH-IBU) and IBP carboxylic acid (CBX-IBU), two IBP metabolites, represent respectively 26 % and 43 % of the total amount of ingested IBP (Ferrando-Climent et al., 2012).

1.3 Wastewater treatment

The wastewater treatment is crucial to protect the environment, to protect the available water, and to prevent the propagation of diseases through water, so preserving and protecting the public health (Machado, 2006). The construction of WWTP serves essentially to treat domestic and industrial wastewaters by removing suspended solids, biodegradable organics, nutrients, pathogenic microorganisms and some priority pollutants, allowing to release the water in safe environmental conditions (Metcalf and Eddy, 2003).

Conventional wastewater treatments generally include preliminary, primary, secondary, and tertiary treatment processes. The preliminary and primary treatments have the task to remove coarse solids that may cause problems in the subsequent steps, as well as settleable organic and inorganic solids and floating materials (Campbell, 2013). The secondary treatment is generally performed by biological methods, where the microorganisms convert complex organic substances (proteins, lipids and carbohydrates) into simpler molecules, carbon dioxide (CO_2) and water (Davis, 2011). Tertiary treatment is employed for specific wastewater constituents that could not be removed by the previous treatments (*e.g.* nitrogen, phosphorus, heavy metals), and also for disinfection. Conventional wastewater treatments are not always sufficient to remove the PPCP, and advanced technological treatments have been proposed, such as Membrane Processes and/or Advanced Oxidation Processes (AOP) (World Health Organization (WHO), 2011). In Table 1, some studies on the removal of pharmaceuticals by conventional treatments are summarized.

The **membrane treatment processes** are used as a separation treatment and are highly efficient to remove micropollutants from the water (Bolong et al., 2009; WHO, 2011). The efficiency of the treatment executed by membranes is dependent of membrane and organic compounds properties, such as molecular weight, polarity, hydrophobicity, chemical nature, pore size, and mechanical resistance (WHO, 2011). Reversed osmose, ultrafiltration and nanofiltration represent membrane filtration processes (WHO, 2011). Reversed osmose is used to remove very small molecules and ions on liquid effluents, by applying pressure, in accordance with the concentration gradient, to the solution on one side of the selective semipermeable membrane (Homem and Santos, 2011). Ultrafiltration and nanofiltration processes are able to remove small molecules, being ultrafiltration more suitable for macromolecules, colloids, most bacteria, some viruses, and proteins; while nanofiltration is more used for small molecules some hardness and viruses (Homem and Santos, 2011; Metcalf and Eddy, 2003). The membrane used in nanofiltration presents a lower pore size (micropores <2 nm) than the membrane used in ultrafiltration, which in turn presents mesopores (2 - 50 nm)) (Metcalf and Eddy, 2003).

Table 1. Removal processes applied in treatment of effluents contaminated with pharmaceuticals.

Compound	Concentration	Type of treatment	Removal efficiency	Reference
CIP Norfloxacin Ofloxacin	30 ng/L	Coagulation/ Flocculation Sand filtration; Granular activated carbon filtration	Norfloxacin and ofloxacin were not affected by coagulation; 30 % of CIP was removed from the effluent. No significant removal was noted regarding these pharmaceuticals (<10%).	(Vieno et al., 2007)
Avilamycin Tylosin	7000 mg/L	Anaerobic Digestion	Reduction varied between 70 and 75 % for Avilamycin; An average of 95 % Tylosin reduction was achieved.	(Chelliapan et al., 2006)
Ibuprofen	1200 - 2679 ng/L	Membrane Biological Reactors	The removal efficiency was: 0 – 100 % for Ibuprofen; 7 – 63 % for Diclofenac; 22 – 44 % for Roxithromycin; 32 – 66 % for Sulfamethoazole; Low efficiency (<14 %) for Carbamaezpine.	(Clara et al., 2005)
Diclofenac	905 - 4114 ng/L			
Roxithromycin	25 - 117 ng/L			
Sulfamethoazole	24 - 145 ng/L			
Carbamaezpine	325 - 1850 ng/L			
Ibuprofen	2600 - 5700 ng/L	Activated sludges	Removal efficiencies about 40 - 65 % for anti-inflammatories (ibuprofen and naproxen), and 60 % for sulfamethoxazole.	(Carballa et al., 2004)
Naproxen	1800 - 4600 ng/L			
Sulfamethoazole	600 ng/L			

The **AOP** are chemical oxidative processes, which have been proposed as promising for efficient degradation of pharmaceutical in water and wastewaters, and can occur through direct reaction, by employing an oxidant, or through indirect reaction through highly reactive secondary species, such as hydroxyl radicals (OH•) (Le-Minh et al., 2010; Verlicchi et al., 2010). However, although they promote a significantly higher removal of the pharmaceuticals contained in the water comparatively to the traditional. However, the AOPs require huge investments and represent considerable operational costs, and in some

cases produce a variety of mutagenic and toxic compounds, thus aggravating the problem (Martins et al., 2016). Moreover, they consume more energy (UV and O_3) and a large amount of chemical reagents (catalysts and oxidizers) (Martins et al., 2016; Moreira et al., 2016; Oller et al., 2011).

1.3.1 Anaerobic Digestion

The wastewater treatment process in WWTP implicates the formation of by-products, namely, primary and secondary sludges, which can be subsequently stabilized in anaerobic digestors. Anaerobic digestion is a mature technology frequently applied to the treatment wastewater or sludges (Leonardo, 2012).

In the absence of molecular oxygen or other alternative electron acceptors, anaerobic microorganisms convert organic substances to biogas, that is essentially composed of methane (CH_4) and CO_2 (Angelidaki and Sanders, 2004; Chan et al., 2009). This biogas holds high quality carburant proprieties for transportation, and can be used in several kinds of energy services (*e.g.* heat, heat combined with electricity) (Ohimain and Izah, 2017). The utilisation of biogas reduces the use of fossil energy, decreasing the environmental impact (*e.g.* global heating, pollution) (Ohimain and Izah, 2017). Lauwers et al*.,* (2013) state that the anaerobic digestion is a robust and efficient technology for the valorisation of different types of organic wastes, and predict that this treatment process will play a crucial part in the future of renewable energy production. This is a complex process, as it requires rigorous anaerobic conditions (oxidation-reduction potential ≤ 200 mV) and depends on the activity of complex microbial communities to transform organic substances into CO_2 and CH_4 (Appels et al., 2008). The anaerobic digestion occurs through four degradation stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis – (Figure 5) (Chen et al., 2014).

The hydrolysis corresponds to the first stage of anaerobic digestion. At this point, insoluble organic substances of high molecular weight (proteins, carbohydrates and lipids) are transformed into soluble organic substances of lower molecular weight (amino acids, sugars, and fatty acids), through the action of the extracellular enzymes excreted by hydrolytic bacteria (Appels et al., 2008; Chen et al., 2008). Lipids hydrolysis is generally a slow process, while carbohydrates are quickly converted into mono and disaccharides and proteins are converted on amino acids in a few days (Deublein and Steinhauser, 2008).

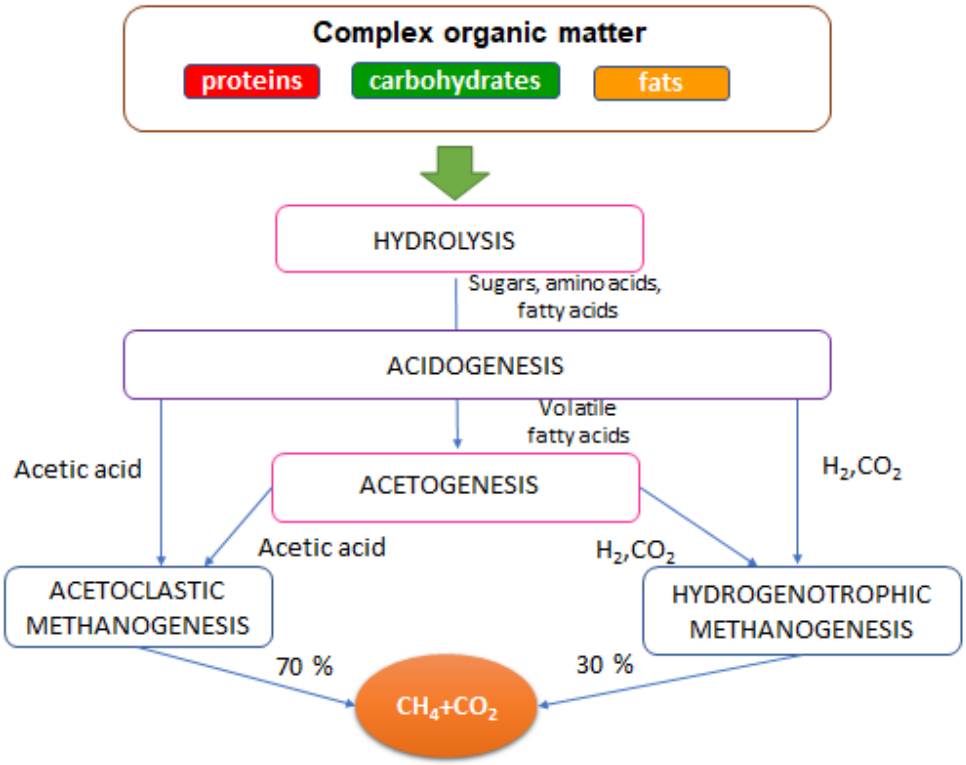

Figure 5. Schematic representation of the main conversion processes in anaerobic digestion. Adapted from Chen et al. (2014).

The second stage of anaerobic digestion is known as acidogenesis. At this step the products deriving from hydrolysis are transformed by the acidogenic bacteria into a variety of products, among these, the organic acids (formic acid, acetic acid, propionic acid, lactic acid, butyric acid), alcohols, NH_3, CO_2 and hydrogen (H_2) (Chen et al., 2014).

During the acetogenesis the products resulting from the acidogenesis are converted into acetic acid, CO_2 and H_2, by the acetogenic syntrophic bacteria or the obligatory H_2 producers, and the transformation of CO_2 and H_2 into acetate, by the homoacetogenic bacteria (Batstone and Jensen, 2011; Mara and Horan, 2003).

The methanogenesis corresponds to the last stage of the anaerobic digestion. This step consists on the conversion of acetate or H_2 and CO_2 by methanogenic archaea, producing CH_4 (Angelidaki et al., 2009; Appels et al., 2008; Mara and Horan, 2003). The acetoclastic methanogenesis is responsible for 70 % of methane generation (Equation 1), using acetate to produce CH_4 (Oliveira, 2012).

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \quad (1)$$

The remaining CH_4 is produced using H_2 as electron donor and CO_2 as electron acceptor by the hydrogenotrophic methanogens (Equation 2).

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (2)$$

Besides the anaerobic oxidation of the organic compounds, reduction of recalcitrant compounds such as azo dyes have been reported to occur under anaerobic conditions, facilitating the biodegradation of these compounds (Pereira et al., 2016).

1.3.2 Parameters influencing the Anaerobic Digestion

The anaerobic digestion is a complex process, considering that its efficiency depends on the existence of a diversified microbial consortium and very specific operational and environmental conditions, as well as specifically favourable anaerobic conditions (Oliveira, 2012). There are several liable parameters influencing the different stages of the anaerobic digestion, among which are the pH, temperature, agitation, nutrients and presence of toxics/inhibitors (Abbasi et al., 2012; Appels et al., 2008).

The **pH** plays an important role in the anaerobic digestion, as it influences the activity of the enzymes. The optimal pH range varies according to the type of microorganism (Angelidaki and Sanders, 2004). As example, for the methanogenic *archaea,* which are highly sensible to the pH of the medium, the most indicated pH range stays between 6.5 and 7.2 (Abbasi et al., 2012; Appels et al., 2008). It has, however, to be considered, that the pH of the medium can be affected by the compounds resulting from the anaerobic digestion processes, such as the production of organic acids, which leads to the diminution of the pH (Appels et al., 2008).

The control of pH in the system can be accomplished through the addition of a buffer solution, that allows to neutralize the production of acids and bases generated throughout the whole process (Batstone and Jensen, 2011). Controlling the pH is extremely important, taking into consideration that if it stays outside of the optimum range the anaerobic process is inhibited, compromising the methane production (Angelidaki and Sanders, 2004).

Just as the pH, the **temperature,** aside from affecting the methane production involved in the anaerobic digestion, influences also the growth rate of the microorganisms and their metabolic activity (Angelidaki and Sanders, 2004; Appels et al., 2008). The ideal temperature for the realisation of the different processes by methanogenic bacteria depends on the microorganism: psychrophilic microorganisms have higher activity at temperature ≤ 20°C; mesophilic microorganisms at temperature in the range of 25 - 40°C and thermophilic microorganisms in the range of 50 - 65°C (Abbasi et al., 2012). The mesophilic microorganisms are the major group of CH_4 producers, and also the most sensitive ones to temperature variations (± 2°C) (Batstone and Jensen, 2011). The majority of the digesters operate under mesophilic conditions (Chen et al., 2014). However, thermophilic conditions offer several advantages compared to the mesophilic conditions, including an increase of the solubility of the organic compounds, an increase of the biochemical reaction rates, and a higher probability for the elimination of pathogens (Appels et al., 2008). On the other hand, it represents some disadvantages such as the costs associated with the necessity to maintain the high temperatures in the reactor and the increase of the ammonia fraction (an inhibitor for the methanogenic microorganisms) (Abbasi et al., 2012; Appels et al., 2008).

The **agitation** is a necessary part of the anaerobic digestion, as it maintains the homogeneity thereby rendering the process more stable (Abbasi et al., 2012). The mixture allows an efficient supply of nutrients to the bacteria, avoiding the concentration of gradients and of temperature, as well (Abbasi et al., 2012; Angelidaki et al., 2009). Nevertheless, the agitation should not be excessive, as it can cause the destruction of aggregates, that are generally important for interspecies relationships within the microbial communities (Abbasi et al., 2012).

The organisms need a large variety of **nutrients** to ensure their development and, consequently, the efficiency and stability of the anaerobic digestion process (Lettinga, 1995; Mara and Horan, 2003). The nutritional requirements are satisfied through the supply of all the essential micro and macroelements for a steady growth of the microorganisms, particularly nitrogen, phosphorous, magnesium, sodium, calcium, cobalt and manganese (Angelidaki and Sanders, 2004). According to Angelidaki et al., (2009) it is very important to guarantee, at least, the necessary levels for the cellular metabolism, to prevent nutrient deficit, but always keeping concentrations that will not cause toxicity. Therefore, it is recommended to ensure that the medium never lacks micro and macronutrients, buffer solution, and vitamins. Limited nutrients lead to poor growth, but it has been demonstrated, that even under limited conditions, a significative reduction of the pollutant load can occur (Lettinga, 1995).

The different microbial groups involved in the anaerobic processes are able to present different responses for one specific toxic substance (Chen et al., 2014). The **inhibition** is generally indicated by the decrease

of the production rate of CH_4 gas, the accumulation of organic acids, or bacterial growth inhibition (Chen et al., 2008). Certain minerals, such as sodium, potassium, calcium, magnesium and sulphur, when at the required concentrations, stimulate the bacterial growth, but, at higher concentrations than the ideal, they can be considered inhibitors (Abbasi et al., 2012). Quite often, the inhibitor compounds are already present in the digester substrate or are generated throughout the digestion (Appels et al., 2008), *e.g.* long chain fatty acids, ammonium, sulphide, and heavy metals (Chen et al., 2014). The pH and temperature can influence the toxicity. For example ammonium (NH_4^+) and NH_3 are generated during the anaerobic treatment through the degradation stage of the nitrogen-based matter, and they can have inhibitor effect at high pH and temperature (Appels et al., 2008; Chen et al., 2014); Heavy metals (e.g. lead, cadmium, copper, zinc, nickel, chromium, and mercury) are not biodegradable and may build up in the wastewaters, turning them potentially toxic, inhibiting metabolic activity, so supressing the CH_4 production (Chen et al., 2014).

1.4 CNT and CM@MNP as redox mediators in Anaerobic Digestion

Pharmaceuticals are resistant to degradation, highly persistent in the environment and potentially capable of causing adverse effects to living organisms, and they have a negative impact on public health (Rivera-Utrilla et al., 2013). The majority of the biological and physical-chemical treatments used to process water are not efficient on the removal of these compounds, requiring an optimisation of these processes or the development of new efficient methodologies (Homem and Santos, 2011).

Concerning anaerobic digestion, one of the limitations is the long start-up, since the growth rate of the methanogenic organisms is low (Chong et al., 2012; Seghezzo et al., 1998). In addition, the reductive transformation of many different recalcitrant compounds proceeds very slowly, requiring high retention times to reach a satisfactory extent, due to electron transfer limitations and to toxicity effects leading to poor performance or even the collapse of anaerobic bioreactors (Van der Zee & Cervantes, 2009; Van Der Zee & Villaverde, 2005). One possible solution for solving these limitations is the use of redox mediators (RM) (Pereira et al., 2014).

RM are compounds, which accelerate the electron transfer from a primary electron donor (substrate) to a terminal electron acceptor (pollutant) to be, degraded (Figure 6). Quinones (*e.g.* anthraquinone-2,6-disulphonate and anthraquinone-2-sulphonate) and flavin-based compounds (*e.g.* flavin adenide mononucleotide and riboflavin) are the most soluble compounds used as RM for azo dye reduction (Santos et al., 2004). Despite these soluble mediators being added at low concentrations, their

continuous addition into the systems is required resulting in increasing costs and continuous discharging of these recalcitrant compounds (Pereira et al., 2010).

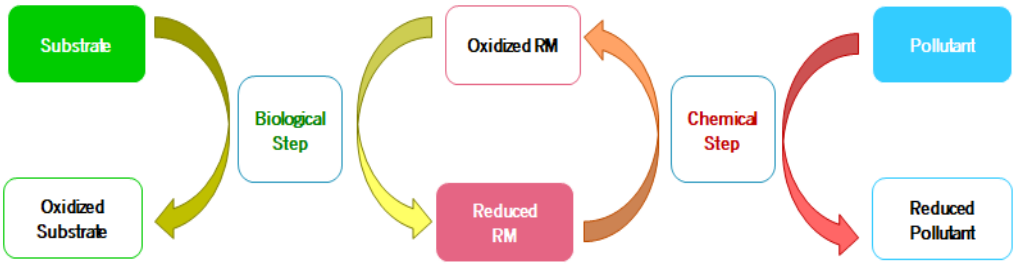

Figure 6. Scheme of the biological and chemical steps involved in the pollutant reduction in the presence of redox mediators. Adapted from Santos et al. (2004).

Insoluble carbon materials (CM) have also been proven to have RM properties. These materials have a high potential for application in the field of environmental biotechnology due to their characteristics that allow them to be utilized as catalysts, namely high specific surface area, pore volume distribution, presence of diverse surface functional groups, good mechanical strength, and the chance of being tailored for specific applications (Pereira et al., 2016; Pereira et al., 2010). In comparison with soluble compounds, these insoluble materials have the following advantages: they can be reused and regenerated, and be easily immobilized inside the reactors, avoiding the need to be fed continuously during the electron shuttling process, so, being effective at low concentrations, decreasing the operating costs (Pereira et al., 2016). Some examples of CM acting as RM on the anaerobic biodegradation of organic compounds (azo dyes and aromatic amines) are the activated carbon; carbon xerogels; carbon nanotubes (CNT) and magnetic CNT (CNT impregnated with 2 % of Fe - CNT@2%Fe) (Pereira et al., 2017, 2014).

Magnetic nanoparticles (MNP) can also be used in environmental applications, as adsorbents, immobilisation agents for microorganisms and enzymes, and as a support for biofilm and water disinfection. They have also been proved as catalysts of many reactions (Ai et al., 2010; Oliveira et al., 2002). Possessing magnetic characteristics gives them the ability to be easily separated by the application of a magnetic field (Pereira et al., 2017).

Combining CM with MNP offers the possibility to create magnetic carbon composites (C@MNP) with synergistic properties, namely the adsorptive and catalytic properties of both and the magnetic character of MNP, improving the material's performance and rendering it easier to be retained and recovered, by applying a magnetic field (Pereira et al., 2017). Some researchers have prepared different C@MNP composites for application on adsorption of contaminants. Recently, C@MNP have been successfully applied for biological and chemical reduction of the azo dye (Acid Orange 10) by Pereira et al., (2017). In this study, different electron transfer mechanisms were proposed: i) the biological oxidation of the co-substrate to the final acceptor; ii) the biological oxidation of the co-substrate to the carbon shell (in core-shell composites) or CNT of the composites and then to the final acceptor; iii) from FeO (Fe^{2+}) of the core of composite to the carbon shell (in core-shell composites) or the Fe (Fe^{2+}) impregnated in CNT to the carbon of the composites and then to the final acceptor. In the case of the abiotic process, only the last mechanism of electron transfer may occur, from FeO (Fe^{2+}) to the carbon of the composite and then to the final acceptor.

1.5 Objetives

This work pretended to evaluate the effect of CM (CNT and a magnetic material CNT@2%Fe) as RM in the anaerobic removal of pharmaceuticals, testing the CIP as model compound. The specific objectives were:

- Evaluate the activity of the anaerobic sludge to be used in the removal assays, testing three different substrates (acetate, ethanol, and mixture of H_2/CO_2 (80:20 % v/v));
- Evaluate the potential toxicity effect of CIP and of the two tested CM, on the activity of the anaerobic sludge;
- Evaluate the efficiency of 0.1g/L of CNT@2%Fe and CNT, as RM in anaerobic removal of CIP;
- Identify possible products resulting from anaerobic reduction of CIP;
- Evaluate the toxicity of the pharmaceuticals, of the CM and of the final treated solutions towards the bioluminescent bacteria *Vibrio fischeri*.

2. MATERIALS AND METHODS

2.1 Chemicals and Materials

2.1.1 Inoculum

Anaerobic granular sludge from a full-scale upflow anaerobic sludge bed (UASB) reactor treating brewery wastewater (near Lisbon, Portugal) was used as inoculum. The volatile solids (VS) of inoculum were 0.08 g/g d.w.

2.1.2 Liquid substrates

As liquid substrates for microorganisms, acetate and ethanol were used. Stock solutions were prepared with a concentration of 3 mol/L, 100-fold concentrated relative to the concentration used in the assays. These stock solutions were stored in the fridge at 4°C.

2.1.3 Pharmaceuticals solutions

CIP was obtained at Sigma-Aldrich, at the purity of 98 %. A stock solution of CIP was prepared in deionized water at a concentration of 50 mg/L. Due to the low solubility of CIP a few drops of hydrochloric acid (2 mol/L) were added, under constant magnetic stirring. This solution was stored at 4°C.

2.2 Carbon Materials: preparation and characterization

The commercial Multiple Wall CNT (MWCNT) sample (Nanocyl 3100) was used without any modification (sample CNT) or impregnated with 2 % of Fe (sample CNT@2%Fe). According to the supplier, the commercial CNT have an average diameter of 9.5 nm, an average length of 1.5 μm and a carbon purity higher than 95 % (Pereira et al., 2014; Tessonnier et al., 2009). Tessonnier et al., (2009) have characterised those MWCNT as having average inner and outer diameters of 4 and 10 nm, respectively. Authors also observed that Nanocyl 3100 contains growth catalyst impurities, mainly Fe and Co (0.19 % and 0.07 %, respectively), sulfur (0.14 %) - probably due to the purification process-, and traces of Al (0.03 %).

CNT@2%Fe were prepared as follows: 2 % wt Fe monometallic catalyst was supported on the CNT by incipient wetness impregnation from aqueous solution of the corresponding metal salt (Fe $(NO_3)_3$). After impregnation, the sample was dried at 100°C for 24 h, heat treated under nitrogen flow at 400°C for 1 h, and finally reduced at 400°C in H_2 flow for 3 h (Pereira et al., 2017). The characterization of this materials is already discribed by Pereira et al., (2017).

2.3 Activity Assays

Activity tests were carried out with liquids substrates (30 mmol/L of acetate or ethanol) and with gaseous substrate (H_2/CO_2, 80:20 % v/v at 1 bar overpressure). Blank assays without substrate addition, or using an inert mixture of N_2/CO_2 (80/20 % v/v) at 1 bar overpressure were also prepared. All the tests were made in triplicate.

For activity tests with liquid substrates, 25 mL vials were used with a working volume of 12.5 mL. For the activity with hydrogen, 70 mL vials were used. The work volume was also 12.5 mL and preparation were similar. The inoculum was added at approximately 3 g VS/L. The medium was prepared as follows: 1 ml/L of resazurin (1 g/L) was added to demineralized water, then the pH was adjusted by addition of hydrochloric acid (HCl, 37 %, analytical grade) or sodium hydroxide (NaOH) (purchased from Sigma-Aldrich). Finally, 3 g/L of sodium bicarbonate were added. Bottles containing the inoculum and the medium were sealed with butyl rubber stoppers and aluminium capsules. Thereafter, the bottles headspace was flushed with N_2/CO_2 (80:20 % v/v), then depressurized, and 100 µL of $Na_2S.9H_2S$ (0.125 mol/L) were added. All the bottles were incubated overnight at 37°C under a rotation of 120 rpm, to deplete the residual substrate.

In the following day, the headspaces were flushed with a mixture N_2/CO_2 (80:20 % v/v at 1 bar overpressure) and the substrate was added, except for the blanks. In the case of liquid substrates, 125 µL of stock solution were added; for the gaseous substrate, the headspace was flushed with H_2/CO_2 (80:20 % v/v, at 1 bar overpressure). The bottles were incubated at 37°C and 120 rpm. The pressure was monitored in each flask, using the pressure transducer technique (Coates et al., 1996; Colleran et al., 1992). In this technique a pressure transducer is used (Centrepoints Electronics, Galway, Ireland) to carry out measurements in sealed vials, thus allowing the monitoring of the pressure variation resulting from the production of biogas over time, caused by the degradation of the different substrates (Angelidaki, et al., 2006). This transducer is able to measure in the range of -200 to +200 mV, with ± 2 atm variations (Centrepoints Electronics, Galway, Ireland).

At the end of these tests, the following analysis were performed for each vial:

- the percentage of CH_4 by gas chromatography (GC) (except for bottles with gaseous substrate and blank gaseous sample);
- the volume of the gas phase, measuring the pressure before and after the injection of 5 mL of air, allowing the determination of the mV / mL ratio;
- the volatile solids.

The activity was obtained by dividing the initial slope of the methane production curve in mL/h by the VS content in each flask, and the values were expressed in $mLCH_4$@STP/ (gVS.day) (Alves, 1998). The values of the blank assays were subtracted to eliminate the influence of the residual substrate present in the inoculum.

2.4 Toxicity Tests

The possible toxic effect of the CIP and carbon materials, CNT and CNT@2%Fe, on the activity of the microbial consortium was evaluated by monitoring the biogas production in sealed bottles, as described previously for the activity tests, with ethanol and H_2/CO_2 as substrate, in the presence of the selected toxicant as can be seen in Figure 7. Blanks (without substrate and inhibitor) and controls (with substrate but without inhibitor) were also prepared. The experimental procedure and the activity calculation were similar to the ones reported for the activity tests. All the assays were performed in triplicate.

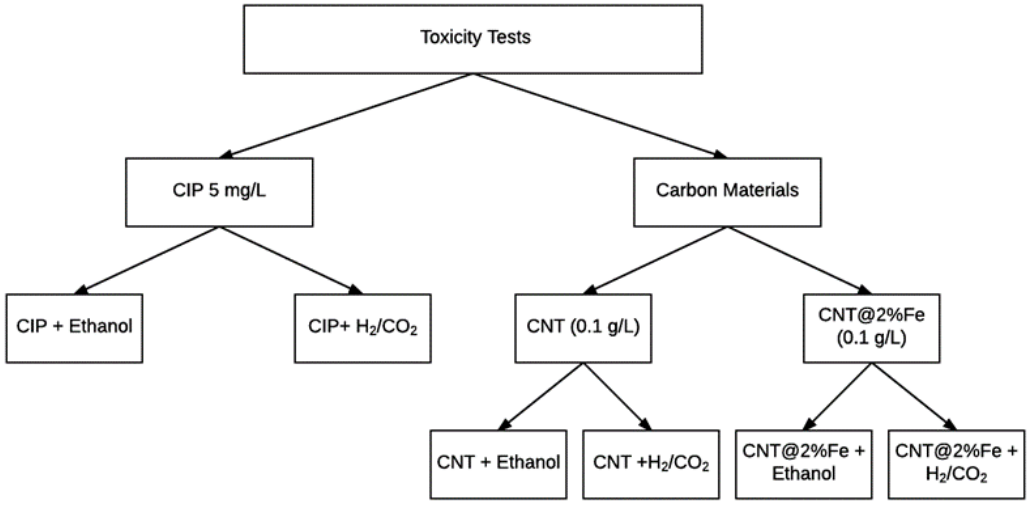

Figure 7. Scheme of toxicity tests with different toxicants.

In the assays with CIP, 1.25 mL of each stock solution were added with a syringe, corresponding to the final concentration of 5 mg/L.

In the assays with the CM, slight differences were introduced in the procedure. In these experiments, the overnight incubation of the sludge at 37°C was performed before preparing the bottles, as the CM are insoluble materials and cannot be added by a syringe. For the assays with liquid substrates, 120 mL bottles were used with a working volume of 50 mL. For the assays with gaseous substrates, on the other hand, 160 mL flasks were used, with a work volume of 35 mL. CNT and CNT@2%Fe were distributed in the bottles, corresponding to final concentration of 0.1 g/L. The medium and the inoculum (approximately 3 g VS/L) were added to the bottles, then the bottles were sealed with rubber stoppers and aluminium caps. From there on, the experimental procedure was similar to the one utilized in the toxicity tests with CIP.

2.5 Biological assays for the removal of Ciprofloxacin

Biological assays with anaerobic sludge were performed aiming to evaluate the efficiency of CNT@2%Fe and CNT as RM in the anaerobic removal of CIP.

2.5.1 Anaerobic medium preparation

The medium used was an adaptation of the anaerobic basic medium (Annex I) described by Angelidaki et al., (2009). The chemicals used to prepare the anaerobic medium solution were purchase from Sigma or Fluka at highest analytic grade purity commercially available.

2.5.2 Experimental Procedure

The biological reductions of CIP were conducted in 200 mL serum bottles, containing 100 ml of work volume. This work volume was composed of medium, inoculum, ethanol, CIP and the CM (CNT or CNT@2%Fe). Ethanol (30 mmol/L) was the primary electron donating substrate. Anaerobic sludge was used as inoculum at a concentration of 3.0 ± 0.2 g/L VS. Biological assays without CM (BA) were also performed as controls, and biological assays without CM and without ethanol (Blanks) were included as well. The effect of CNT and CNT@2%Fe, on biological reduction (BA + CNT and BA + CNT@2%Fe, respectively) was tested at a concentration of CM 0.1 g/L. This concentration is in accordance with other works (Pereira et al., 2010). CIP was added at the final concentration of 5 mg/L. This concentration was chosen in order to facilitate its detection and the study of removal kinetics. Firstly, the sludge was

incubated overnight at 37°C in a rotary shaker at 120 rpm in order to consume all the residual substrate. After this period, the CM, the medium and the sludge were added to the bottles. They were then sealed with a rubber stopper and an aluminium cap and flushed with N_2/CO_2 (80:20 % v/v). Thereafter the CIP and ethanol were added with a syringe from the stock solution up to the desired concentration.

Abiotic controls i.e., controls without sludge, with different CM were also included, and were coded as Ab Control CNT and Ab Control@CNT2%Fe. These controls were done in the same conditions than biological controls but in the presence of ethanol.

All experiments were prepared in triplicate and were incubated at 37°C and 120 rpm over the entire assay. CIP and ethanol concentrations were monitored during 24 h by HPLC (High Performance Liquid Chromatography) as described below.

At the end of 24 h reaction, samples taken from the different removal assays and controls were further analysed for toxicity with *V. fischeri*.

2.6 Analysis

2.6.1 Solid Content

The total and volatile solids were determined gravimetrically according to the method described in *Standard Methods* (APHA et al., 1999). The total solid content (TS) was determined after evaporation at approximately 105°C for 24 h in the oven. The volatile solids (VS) were determined after the sample being calcined at high-temperature (550°C).

2.6.2 Composition of the gas produced

The concentration of CH_4 present in the biogas was determined by GC, using a Shimadzu GC-2014 gas chromatograph fitted with Porapak Q 80/100 mesh, packed stainless-steel column (2 m x 1/8 inch, 2mm) and a flame ionization detector (FID). The column, injection port and detector temperatures were respectively 35, 110 and 220°C. Nitrogen was the carrier gas at a flow rate of 30 mL/min. Headspace gas was sampled by a 500 µL pressure-lock syringe (Hamilton). The values of CH_4 production were corrected for the standard temperature and pressure conditions (STP). Initially, standard CH_4 was injected (with 40 % CH_4) and, afterwards the samples were also injected. Samples and standard injections were done in triplicate.

2.6.3 HPLC analyses

The removal of CIP was analysed by HPLC. Samples were collected at different reaction times (0, 0.5, 1, 3, 5, 8 and 24 h), then centrifuged for 10 min at 10.000 rpm to remove sludge and CM, and the supernatants were filtered with Spartan 13/0.2 RC filters, Whatman 0.2 μm pore size. A calibration curve was made by analysing CIP solutions at concentrations between 0.5 and 5 mg/L (Annex III). HPLC analyses were performed in a HPLC (Shimadzu Nexera) equipped with a Diode Array Detector (SPD-M20A), an autosampler (SIL-30AC), degassing (DGU-20A5R) an oven (CTO-20AC), communication bus module (CMB -20A) and LC -30AD, and Labsolutions software. A RP-18 endcapped Purospher Star column (250×4 mm, 5 μM particle size, from MERK, Germany) was used. The mobile phase was composed by the solvents: **A**, 0.1 % Formic Acid solution (purchased from Merk) and **B**, ACN. The compounds were eluted at a flow rate of 0.8 mL/min and at 40°C, with the following gradient: 5–15 % B (up to 6 min), 15 % B (up to 18 min), 15–40 % B (up to 30 min), 40 % B (up to 40 min), 40–5 % B (up to 43 min), 5 % B (up to 50 min). The injection volume was 10 μL. CIP was monitored at 275 nm. The retention time (Rt) of CIP was 12.2 min.

The percentage of pharmaceuticals removal (PR) was calculated according to equation:

$$PR(\%) = \left(\frac{C_0 - C_t}{C_0}\right) \times 100 \quad (3)$$

Where:

C_0: the initial pharmaceutical concentration;

C_t: the pharmaceutical concentration at selected time (t);

First order reduction rate constants were calculated in OriginPro 6.1. software, applying the equation 4:

$$C_t = C_0 + C_i e^{-kt} \quad (4)$$

Where:

C_t is the concentration at time t;

C_0 the offset;

C_i is the concentration at initial time;

K, the first order rate constant (d^{-1}) and t is the accumulated time of the experiment.

The ethanol consumption and formation of acetate was also analysed by HPLC (Jasco, Japan), equipped with RI detector (Jasco 2031) and UV detector (Jasco UV-2070 plus), using a Rezex ROA Organic Acid H+ (300 mm x 7.8mm) column. The elution was made at 60°C using sulfuric acid (0.0025 mol/L) as mobile phase, at a flow rate of 0.6 mL/min.

2.7 Toxicity assays with *Vibrio fischeri*

2.7.1 Growth and preparation of the *Vibrio fischeri*

V. fischeri strain NRRL-B-11177 "BioFix® Lumi luminous bacteria" was obtained by Macherey-Nagel GmbH & Co. KG (Düren, Germany), in freeze-dried form and grown in laboratory. V*. fischeri* was grown under aerobic conditions in a growth medium for bioluminescent bacteria as described in the international standard ISO 11348-1 (2007). The pH of the agar and liquid medium was adjusted to 7.0 ± 0.2, with HCl (1 mol/L) or NaOH (1 mol/L) (HI 207 Bench pH meter for Education from HANNA Instruments) and the sterilization was performed by autoclaving at 15 lbs pressure (121°C) for 20 min. *V. fischeri* was transferred from cryovials to petri dishes with solid growth medium and incubated during 3 days at 20 ± 1°C. A luminescent colony, identified in the dark by visual observation, was inoculated in an Erlenmeyer flask (250 mL) containing 50 mL of growth medium and incubated at 20 ± 1°C, in an orbital shaker at 180 rpm. After 24 h of incubation period, the OD at 578 nm (OD_{578}) was adjusted to 0.6 and the luminescence were monitored in a microplate reader (Biotek® Cytation3), using a black 96-Well Optical-Bottom Plate with Polymer Base (Nalge Nunc™ International). The grown culture was centrifuged in Beckman Allegra 64R at 6000 ± 2000 rpm for 5 minutes. The supernatant was discarded, and the pellet resuspended in sodium chloride (NaCl) 2 % solution (obtained by Panreac).

2.7.2 Samples Preparation

This step was divided into the preparation of the bacteria suspension tests and the preparation of the samples for the toxicity assessment (CIP solution, solutions after 24 h of treatment: of BA, BA + CNT, BA + CNT@2%Fe, Ab Control CNT and Ab Control@2%Fe). In order to evaluate the effect of CM, solutions containing 0.1 g/L of CNT and 0.1 g/L of CNT@2%Fe, were prepared in an anaerobic medium, similar to that of the biological assays, with a final volume of 100 mL. After 24 h of incubation, samples were centrifuged as well and toxicity evaluated. The possible toxic effect of the anaerobic medium was also analysed. For the negative control, 2 % NaCl, and a bacteria suspension were used. On the other hand,

the positive control was prepared with heptahydrate zinc sulphate ($ZnSO_4.7H_2O$, obtained by Panreac), at a 19,34 mg/L concentration, being extremely toxic for *V. fischeri* (Heinlaan et al., 2008).

V. fischeri is a marine bacterium, thus the salinity of the samples is an important factor for toxicity assays in order to maintain the good conditions for luminescence emission. So, the salinity of the samples was adjusted to 2 % NaCl, using a NaCl 20 % solution. These bacteria are also very sensitive to temperature, being crucial to ensure a non-variable temperature of 20 ± 2°C. pH, oxygen concentration and turbidity were also verified and rectified when necessary.

2.7.3 Toxicity Assessment

The toxicity assays were made analysing the variations of the bacteria's luminescence when they get in contact with the tested samples.

For implementation of the assays, 100 µL of the sample to be tested and 100 µL of bacteria test suspension were mixed in a black 96-Well Optical-Bottom Plate with Polymer Base, and the luminescence readings were made with a microplate reader (Biotek® Cytation3). The Biotek® Cytation3 was used in kinetic mode. This allows the continuous reading of the luminescence emission by *V. fischeri* during the test.

The luminescence inhibition was analysed after 30 min.

The toxicity evaluation of the samples is based on the luminescence inhibition (INH %) caused by the presence of potentially toxic samples to bacteria:

$$INH\ \% = 100 - \left(\frac{IT_t}{KF \times IT_0}\right) \times 100 \quad (5)$$

$$\text{With } KF = \frac{IC_t}{IC_0} \quad (6)$$

Where:

KF is the correction factor and characterizes the natural loss of luminescence of the control;

IC_0 is the initial luminescence intensity of the control sample;

IC_t the luminescence intensity of the control after contact time, t;

IT_0 the Luminescence intensity of test sample, immediately before the addition of the tested sample at the time 0;

IT_t is luminescence intensity of the sample after the contact time.

All the factors are represented in relative light units (RLU). The inhibition is calculated after the exposure of 30 min (Heinlaan et al., 2008; ISO 11348-3, 2007).

The toxicity assays with *V. fischeri* include the initial CIP solution (5 mg/L), solution with CM and the treated solutions (24 h of reaction) in all the conditions tested.

3. RESULTS AND DISCUSSION

3.1 Activity of the anaerobic sludge

In order to evaluate the activity of the microbial populations in the granular anaerobic sludge, activity tests were carried out on three different substrates: acetate, ethanol and H_2/CO_2 (Table 2). Acetate and H_2/CO_2 were used as direct substrates for methanogenesis (acetoclastic and hydrogenotrophic, respectively). Ethanol was also studied as substrate, taking into consideration the origin of this sludge (a brewery WWTP).

The anaerobic sludge exhibits practically no activity in acetate (Table 2), considering that ideally specific methanogenic activity with acetate should be higher than 100 mL CH_4STP/gVS.day (Angelidaki et al., 2009). Good activity was verified with H_2/CO_2 and ethanol (Table 2). The fact that the inoculum does not have any activity in acetate can be an indication that it consists mainly of hydrogenotrophic rather than acetoclastic methanogens.

Table 2. Activity of Anaerobic Granular Sludge

	Specific Activity mL CH_4@STP/(gVS.day)		
Substrate	Acetate	Ethanol	H_2/CO_2
Anaerobic Granular Sludge	4.5 ± 2.4	89.7 ± 21.6	417.7 ± 20.7

3.2 Toxicity of Ciprofloxacin and Carbon Materials

For the anaerobic removal assays, the chosen concentrations of CM and CIP were 0.1 g/L and 5 mg/L, respectively. This CIP concentration is high when compared to the real concentrations present in wastewaters (ng/L up to µg/L), but it was dictated by analytical limitations, *i.e.* by the lower detection limit obtained in the HPLC (0.3 mg/L). By using this concentration, it was possible to achieve the objectives of this work, that is to monitor the kinetics of CIP removal and the effect of CM as electron shuttles of the process. The implementation of the process at real scale will imply concentration of the

samples. For this reason, the toxicity of CIP was evaluated to understand if higher concentrations compromise the viability of the removal process, and this was also the case for the CM.

For the toxicity assays with ethanol and 5 mg/L of CIP, as tested, the CIP inferred a toxicity of approximately 45 % to the microbial community (Annex II). For the toxicity assays with H_2/CO_2 and 5 mg/L of CIP, as tested, the CIP was not toxic to the hydrogenotrophics (Annex II).

For the toxicity assays with CM, 0.1 g/L of CNT did not infer toxicity in ethanol. On the other hand, when H_2/CO_2 was used as substrate instead not only no toxicity was noted but also an increase of methanogenic activity was observed, which agrees with the reported stimulation of pure cultures of methanogens by MWCNT (Salvador et al., 2017). Interestingly, when 0.1 g/L of CNT@2%Fe was used instead, an increase of activity in both substrates was shown (Annex II).

3.3 Removal of Ciprofloxacin

The reduction of CIP under biologic and abiotic conditions was followed by HPLC over 24 h, time at which the equilibrium was reached and no further decrease of CIP concentration was observed. Figure 8 presents the chromatographs obtained from the analysis of the different samples at 0, 5, 8 and 24 h.

The results show the presence of CIP at 275 nm with a retention time (Rt) of 12.2 min. Through the chromatograms analysis, it was possible to observe the decrease of the CIP peak over the time of reaction, in the different assays. After performing a spectral scan within a wavelength of 190-300 nm, a new peak at a Rt of 4.2 min was detected and increased with the time of reaction (Annex IV, figure IV.1). However, that peak was also detected in the absence of CIP, meaning that it is not a by-product of CIP degradation (Annex IV, figure IV.2). This result suggests that it may be assigned to a metabolic product resulting from the conversion of ethanol, possibly acetate. This hypothesis was confirmed by HPLC analysis of different acetate concentrations (Annex IV, figure IV.3).

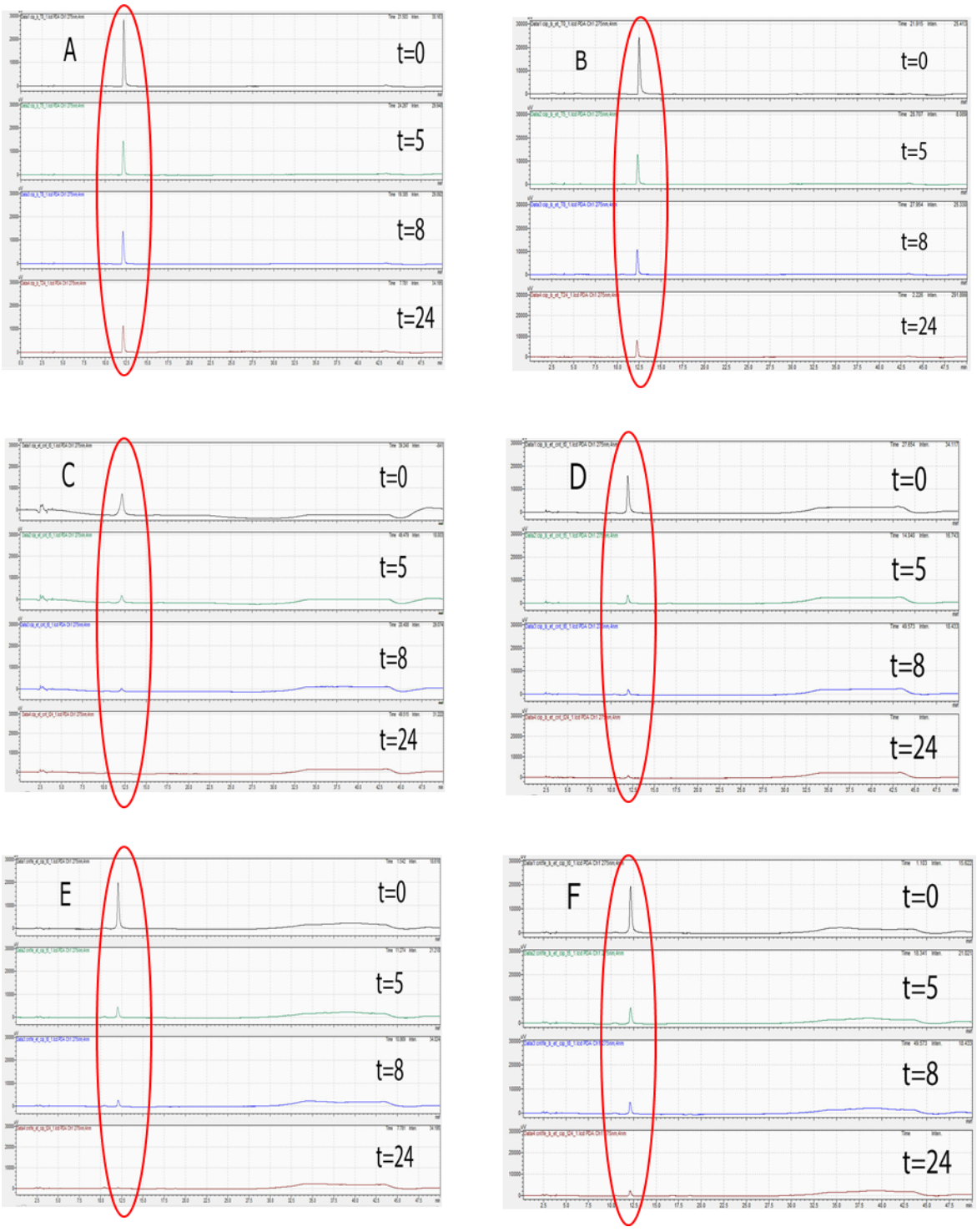

Figure 8. HPLC chromatograms at 275 nm of the CIP removal assays: **(A)** Blank; **(B)** BA; **(C)** Ab control CNT; **(D)** BA + CNT; **(E)** Ab Control CNT@2%Fe; and **(F)** BA + CNT@2%Fe.

As can be seen in Figure 9, the reactions followed the first order kinetics. The extent and rates of CIP reduction at the different conditions were calculated and the results are set in Table 3.

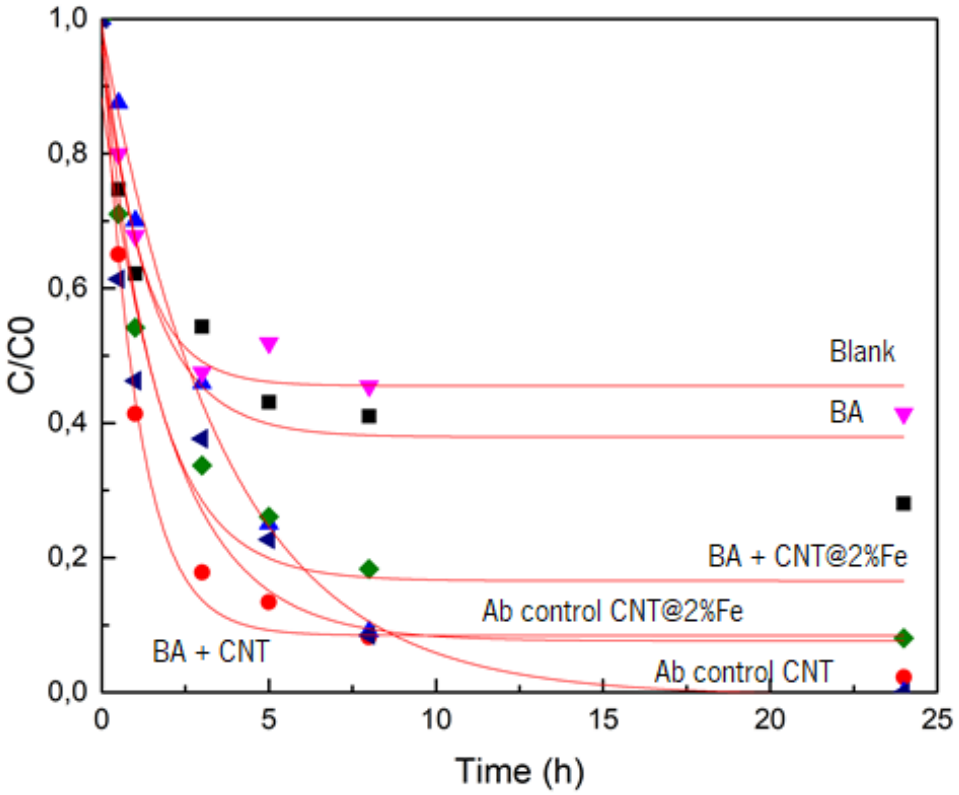

Figure 9. First order rate curves of CIP: (▼)Blank; (■) BA; (●) BA + CNT; (♦) BA + CNT@2%Fe; (▲) Ab control CNT; (◀) Ab control CNT@2%Fe.

Table 3. Effect of CM (CNT and CNT @2%Fe, 0.1g/L) on the extent (%) and rates (d^{-1}) of CIP (5 mg/L) removal.

Assay	Removal (%)	Rate (d^{-1})
Blank	59 ± 2	24 ± 1
BA	72 ± 2	19 ± 4
BA+ CNT	98 ± 1	24 ± 1
BA + CNT@2%Fe	92 ± 1	16 ± 2
Ab Control CNT	100 ± 1	7 ± 1
Ab Control CNT@2%Fe	100 ± 1	12 ± 6

In the BA, biological assay in the absence of CM, CIP removal was approximately 72 %. However, in blank assay in which ethanol was not added CIP removal was approximately 59 %, which suggests a high

adsorption of CIP to sludge. The difference between these two values suggests the contribution of biological activity for CIP removal in BA.

In the presence of CNT and CNT@2%Fe, CIP removal were higher 98 % and 92 %, respectively. This increase can be related to the CIP adsorption to the materials or a possible stimulation of the biological activity by the CM, or both. Indeed, in the abiotic controls (Ab control CNT and Ab control CNT@2%Fe), there is a complete removal of CIP. This removal is likely due to the adsorption of CIP to the materials.

In short, our results suggest different removal mechanisms of CIP (Figure 10): adsorption to sludge and/or different CM and its biological removal, however, this last mechanism needs to be verified by complementary analysis (*e.g.* Liquid Chromatography – Mass Spectrometry).

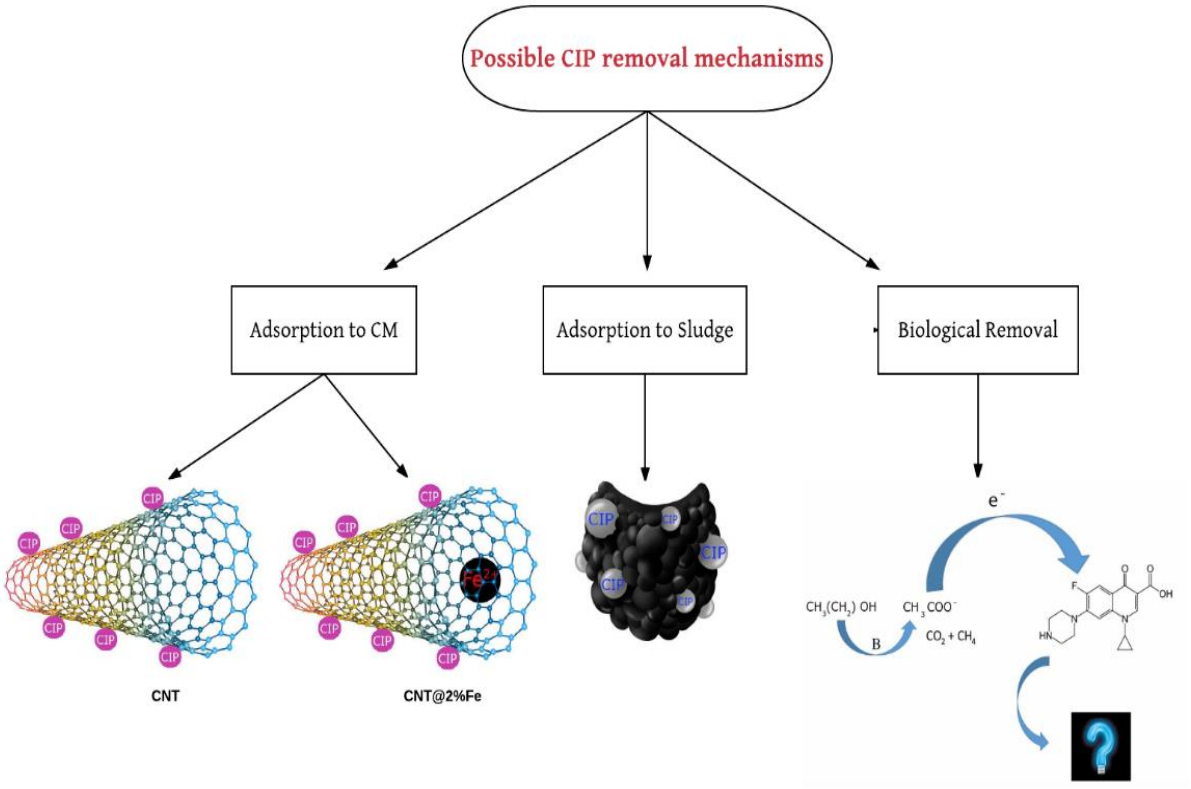

Figure 10. Possible CIP removal mechanisms.

Consumption of ethanol was verified by HPLC and confirmed the occurrence of biological activity. Table 4 shows the values maximum ethanol removal (in percentage) and the removal rate for different reduction assays. Additionally, the amount of acetate formed in biotic assays was also determined.

Table 4. Removal of ethanol (%) and rates (d^{-1}) in biological and abiotic CIP (5 mg/L) removal assays. Ethanol (30 mmol/L) was used as substrate and the effect of CM in its removal was evaluated.

Assay	% Removal	Rate (d^{-1})	Acetate (mmol/L)
BA	95 ± 4	0.996 ± 0.138	26 ± 1
BA+ CNT	95 ± 5	0.912 ± 0.058	25 ± 1
BA + CNT@2%Fe	93 ± 2	0.827 ± 0.014	25 ± 1
Ab Control CNT	0	0	n.a
Ab Control CNT@2%Fe	0	0	n.a

n.a. – not applicable

The ethanol was almost totally consumed (almost 95 %) in the biological assays, both in the presence (BA+ CNT and BA + CNT@2%Fe) and in the absence of CM (BA) (Table 4). According to the literature, one mole of ethanol can be converted in one mole of acetate and two moles of hydrogen (equation 7) (Deublein and Steinhauser, 2008).

$$CH_3(CH_2)OH + H_2O \rightarrow CH_3COOH + 2H_2 \qquad (7)$$

During the test, the anaerobic sludge consumes the ethanol and produces acetate. As can be seen in Figure 11, as the ethanol decreases the amount of acetate increases over time. At the end of the 24 h assay, propionic acid was also detected at a concentration of approximately 2 mmol/L (data not shown).

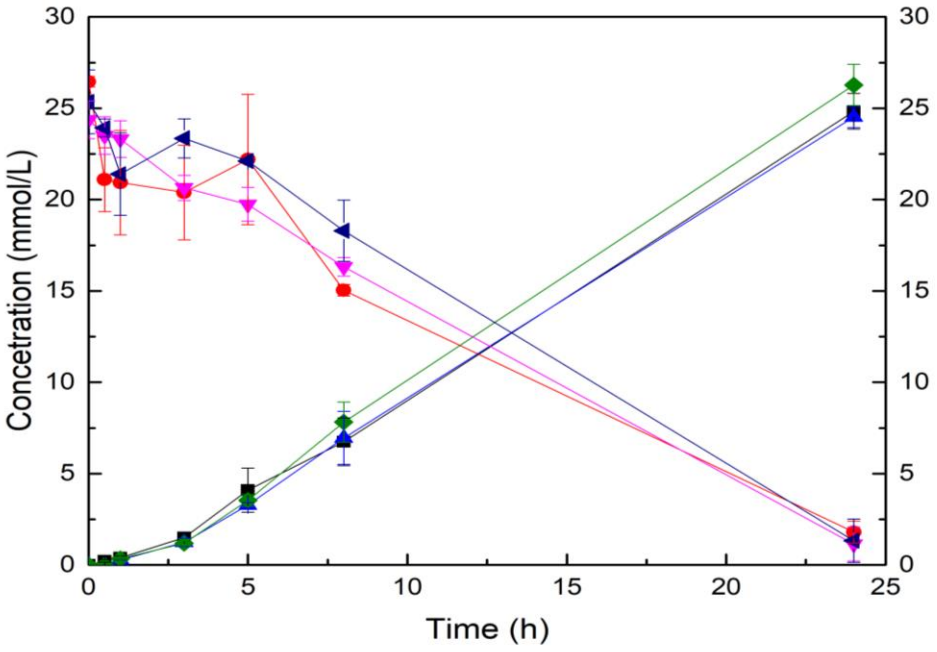

Figure 11. Degradation of ethanol by anaerobic sludge in different assays: (▼) BA;(◀) BA + CNT; (●) BA + CNT@2%Fe. Acetate production in the assays: (▲) BA; (♦) BA + CNT; (■) BA + CNT@2%Fe.

For the abiotic assays, as expected, there was no ethanol removal. However, due to the presence of CM, a small adsorption of ethanol occurs at the beginning (approximately 30 %), followed by its desorption, getting back to the initial value.

3.4 Toxicity of Ciprofloxacin or its by-products towards *Vibrio fischeri*

Evaluation of the toxicity of the samples collected at the end of the anaerobic removal assays is crucial to understand if the toxicity of CIP decreases after the treatment, in order to verify if the possible by-products could presumably infer toxicity. The same method was used to study the contributions of CM to the toxicity effect of the treated solutions.

The toxicity is evaluated according to the percentage of luminescence inhibition of *V. fischeri* when exposed to toxic substances. To assess the toxic effect of these, negative (bacteria in the absence of the toxicant) and positive ($ZnSO_4.7H_2O$) controls were conducted (Heinlaan et al., 2008).

Table 5 presents the values of luminescence inhibition after 30 min of contact between the bacteria and the different samples, namely positive controls, initial CIP solution, anaerobic medium, solutions that were previously exposed to the CM, and samples of the different CIP treatments, collected at the end of 24 h reaction.

Table 5. Percentage of *V. fischeri* luminescence inhibition in all tested samples, after 30 min of exposure

Samples	% inhibition
Positive Control ($ZnSO_4$)	83 ± 8
CIP (5 mg/L)	56 ± 10
Anaerobic medium	0
Medium exposed to 0,1 g/L CNT	28 ± 1
Medium exposed to 0,1g/L CNT@2%Fe	35 ± 14
Treated CIP samples -Abiotic Assays	
Ab control CNT	15 ± 9
Ab control CNT@2%Fe	26 ± 7
Treated CIP samples -Biological Assays	
BA	30 ± 4
BA +CNT	19 ± 8
BA + CNT@2%Fe	26 ± 7

The obtained results indicate that, after 30 min of contact with the bacteria, the inhibition of luminescence caused by CIP solution was approximately 56 %. When the *V. fischeri* luminescence is decreased at levels between 30 % and 50 %, the sample is considered "slightly toxic". However, if the extent of inhibition is above 50 %, the sample is considered "toxic or very toxic" for this microorganism (Mendonça *et al.*, 2009). Therefore, it can be concluded that the CIP solution is toxic.

The samples collected at the end of the BA present an inhibition of luminescence of about 30 %, which reflects its slightly toxic character. These 30 % of luminescence inhibition can be related to the amount of CIP existing in the treated solution (as CIP was only partially removed in this assay) or to the possible by-products. When compared to the CIP solution, it was verified that the treatment led to a reduction in luminescence inhibition of around 26 %.

For the treated solutions of BA + CNT and BA + CNT@2%Fe the inhibition of luminescence was similar to that obtained in control solutions previously incubated with the materials. The same was verified for the abiotic assays in the presence of these materials. As both assays presented a higher percentage of CIP removal, the inhibition of luminescence may be associated with the presence of CM, *i.e.*, the treated solutions may contain traces of small amorphous materials from CM or even impurities that were not retained in filters.

4. CONCLUSIONS AND FUTURE PERSPECTIVES

The main goal of this project was to evaluate the effect of CM, CNT and CNT@2%Fe, as RM in anaerobic reduction of CIP. For this purpose, the sludge activity in different substrates was evaluated. Besides, the toxic effect of CIP and CM was also evaluated when in contact with anaerobic sludge. Afterwards, biological assays were performed in order to remove the CIP in the presence of both CM. Finally, the treated solutions obtained from these experiments were submitted to toxicity assays with *V. fischeri.*

The results revealed that the sludge did not have activity in acetate. However, the sludge exhibited good activity in ethanol and H_2/CO_2. Toxicity tests revealed that CIP inhibited 45 % of the anaerobic microbial community growing in ethanol, but it did not present toxicity to the hydrogenotrophic methanogens. The CM did not present toxicity regardless of the substrate used.

The application of low concentrations of the different CM in the biological treatment of the CIP solution increased the removal of CIP. The extent of CIP removal in the assays without CM (≈72 %) was lower than in the presence of CM (> 90 %), thus proving that the presence of CM improves the efficiency of this treatment process. Better results were obtained with CNT than with CNT@2%Fe. Our results suggest the occurrence of different CIP removal mechanisms, namely adsorption to sludge and/or different CM and biological removal.

The CIP solution was demonstrated to be toxic as evaluated with the standard method ISO 11348-1 (2007). However, all treated solutions were considered only slightly toxic and was attributed to the CM. Thus, it can be concluded that the use of these materials may be advantageous since they increase the efficiency of CIP removal and detoxification after the proposed treatment was achieved.

Aiming to conclude the work discussed in this thesis, as future work it is essential to identify the possible by-products of CIP removal by Liquid Chromatography – Mass Spectrometry in order to confirm the biological removal of CIP and to understand the possible mechanisms involved in these reactions.

As a future perspective, other materials, as for instance, CM impregnated with increased amount of iron impregnated or other modifications of CNT, could be tested as RM. Other pharmaceuticals can also be evaluated with this type of treatment to understand if this method of treatment is applicable to general degradation of pharmaceuticals. Application of the process in continuous is important aiming to achieve the full-scale application.

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ANNEX I – ANAEROBIC MEDIUM

Table I.1. Anaerobic basic medium. Adapted from (Angelidaki et al., 2009)

Description of Anaerobic basic medium
The basic medium is prepared from the following stock solutions (in distilled water):
Solution A: 100 g/L NH_4Cl; 10 g/L NaCl;10 g/L$MgCL_2.6H_2O$; 5 g/L $CaCl_2.2H_20$;
Solution B: 200 g/L K_2HPO_4;
Solution C (trace-metal and selenite solution): 2 g/L $FeCl_24H_20$; 0,05 g/L H_3BO_3; 0,05 g/L $ZnCl_2$; 0,038 g/L $CuCl_2$ $2H_20$; 0,05 g/L $MnCl_2$ $4H_2O$; 0,05 g/L $(NH_4)_6Mo_7O_{24}$ $4H_2O$; 0,05 g/L $AlCl_3$; 0,05 g/L $CoCl_2$ $6H_2O$; 0,092 g/L $NiCl_2$ $6H_20$; 0,5 g/L Ethylenediaminetetracetate;1 ml concentrated HCl; 0,1 g/L NA_2SeO_3 $5H_20$;
Solution D (vitamin mixture): 2 mg/L biotin; 2 mg/L folic acid; 10 mg/L pyridoxine; 5 mg/L ridoflavin; 5 mg/L thiamine hydrochloride; 0,1 mg/L cyanocobalamine;5 mg/L nicotinic acid:5 mg/L P-aminobenzoic acid;

ANNEX II –TOXICITY TESTS

The sludge activity in ethanol and H_2/CO_2, in the presence of the different toxicants (CIP, CNT and CNT@2%Fe), is presented in Table II.1, as well as the toxicity expressed as percentage of inhibition in each assay.

Table II.1 Activity and percentage of inhibition from the different toxic agents: CIP and CM (CNT and CNT @ 2% Fe).

	Activity (mL CH_4@STP/(gVS.day))		% inhibition	
	Ethanol	H_2/CO_2	Ethanol	H_2/CO_2
Pharmaceuticals				
Control (no toxicant)	20 ± 2	458 ± 55		
CIP (5mg/L)	9 ± 1	479 ± 41	≈ 45	≈ -4
Carbon Materials				
Control CNT	18 ± 1	275 ± 15		
CNT (0.1 g/L)	19 ± 2	350 ± 22	≈ -1	≈ -27
Control CNT@2%Fe	20 ± 1	475 ± 41		
CNT@2%Fe (0.1g/L)	30 ± 1	516 ± 13	≈ -50	≈ -9

* Negative values of % inhibition correspond to a stimulus of the activity

ANNEX III – CIPROFLOXACIN CALIBRATION CURVE AS MONITORED BY HPLC

The CIP calibration curve, shown in Figure III.1, was constructed using solutions with different CIP concentrations between 0.5 mg/L and 5.0 mg/L and area values given by HPLC analysis: y=421755.114x-10714 ($R^2 = 0.997$) where the value of x corresponds to the CIP concentration and y corresponds to the peak area obtained in the HPLC, for the corresponding concentration, at 275 nm. As expected, the higher the concentration, the larger the peak area. The R^2 close to 1 means that the calibration curve can be considered reliable, since the coefficient is close to the unit, translating into a good linearity between the two analysed variables.

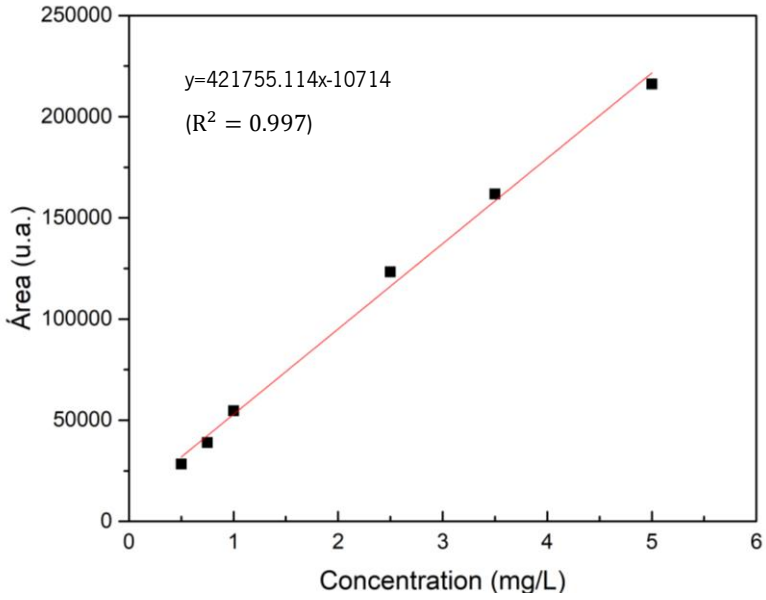

Figure III.1. CIP calibration curve as obtained by HPLC.

ANNEX IV – IDENTIFICATION OF PEAK AT RT 4.2 MIN OBTAINED IN HPLC CHROMATOGRAMS OF CIPROFLOXACIN REMOVAL, AS MONITORS AT 190-300 NM

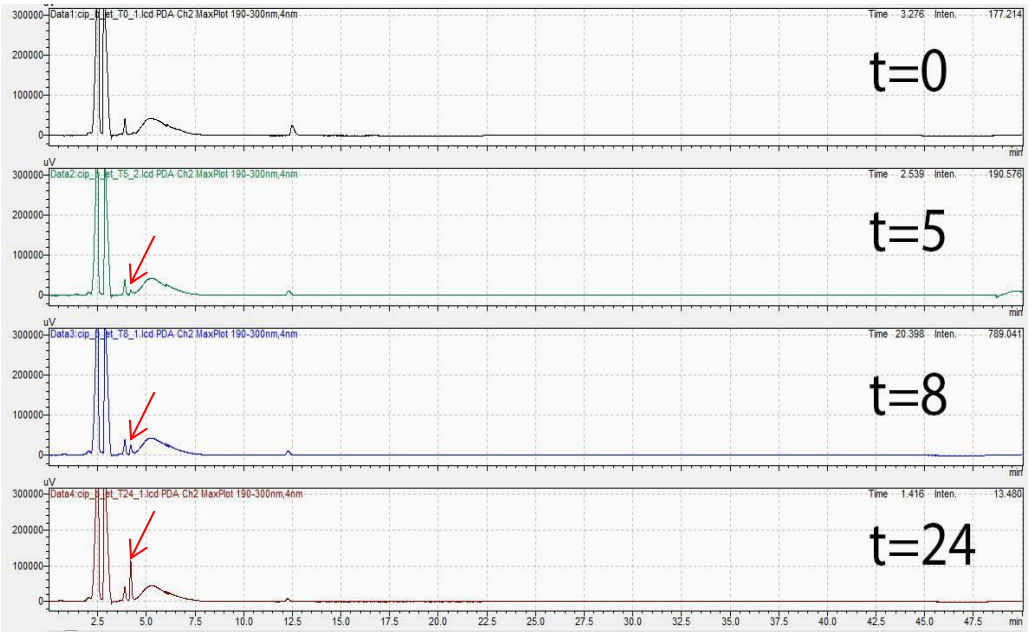

Figure IV.1. HPLC chromatograms of biological assay without CNT (BA) at 190-300 nm. Increase of the peak at Rt 4.2. min, assigned to acetate.

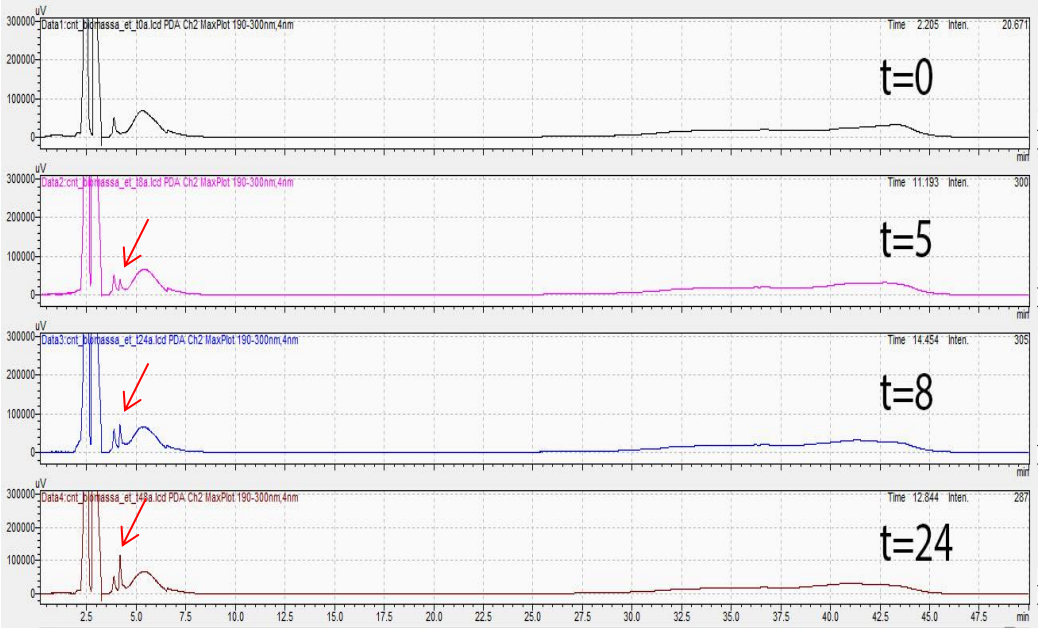

Figure IV.2. HPLC chromatograms of biological assay without CIP at 190-300 nm. Increase of the peak at Rt 4.2 min, assigned to acetate.

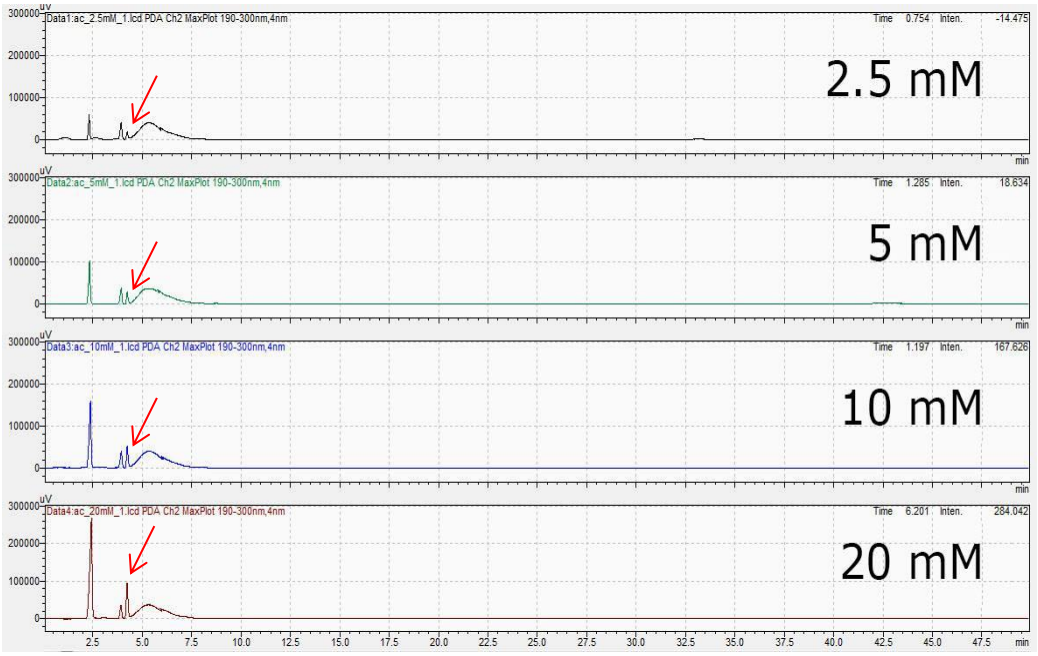

Figure IV.3. HPLC analysis of different concentrations of acetate at 190-300 nm.

ANNEX V– ETHANOL CALIBRATION CURVE AS MONITORED BY HPLC

The ethanol calibration curve, shown in figure V.1, was constructed using solutions with different concentrations of ethanol, between 10 mM and 60 mM and area values given by HPLC analysis.The equation obtained by this curve is: y = 3132.1x + 1395,9, where the value of x corresponds to the concentration of ethanol and the y corresponds to the peak area obtained in the HPLC of corresponding concentration.

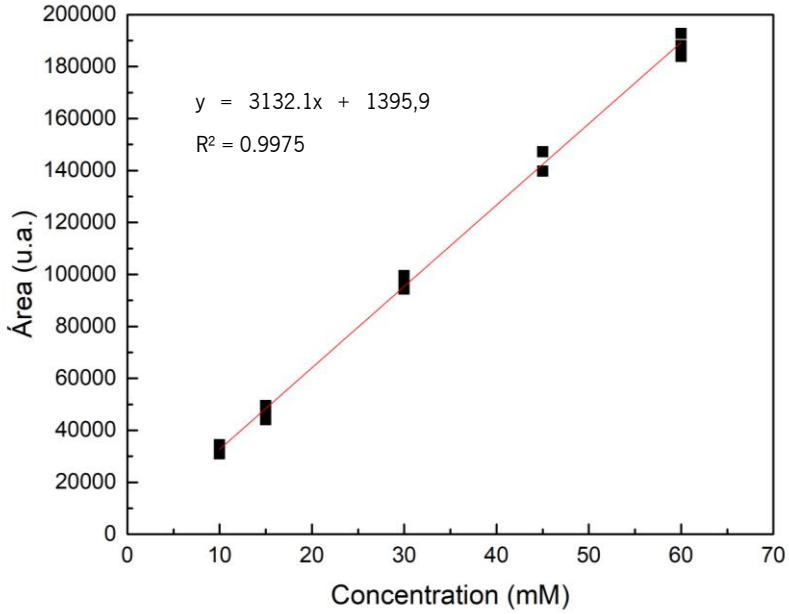

Figure V.1. Ethanol calibration curve by HPLC.