Universidade do Minho
Escola de Engenharia

Juliana Patrícia Rodrigues Pereira

Application of Carbon Nanomaterials in Wastewater Treatment

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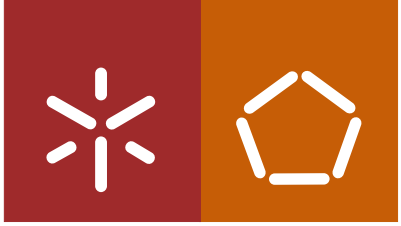

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Application of Carbon Nanomaterials in Wastewater Treatment

Master Dissertation
Masters in Biotechnology

Work supervised by:
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Muito obrigada a todos!

Estes anos são viagem...

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

A poluição das águas é um dos mais sérios problemas em todo o mundo. A rápida industrialização e o aumento significativo da população levam à contaminação dos recursos de água com diferentes micropoluentes, tais como, corantes azo e fármacos.

As atuais tecnologias de tratamento de águas residuais não são efetivas na redução destes compostos, resultando na sua disseminação nos solos, terrenos agrícolas, águas superficiais e subterrâneas e até na água potável, resultando em efeitos negativos para a saúde pública e na vida aquática. Tratamentos anaeróbios têm sido descritos para a biodegradação de micropoluentes, contudo estas reações decorrem lentamente devido à natureza recalcitrante dos compostos, sendo uma limitação para a aplicação dos bioprocessos anaeróbios. Deste modo, é adicionada à reação materiais ou compostos que atuam como mediadores redox (RM), de modo a acelerar a reação de degradação, pretendendo-se ultrapassar esta barreira. Vários materiais de carbono (CM) têm sido descritos como bons RM para a redução de diferentes micropoluentes.

Neste trabalho, diferentes nanotubos de carbono (CNT) com modificações na química da superfície, nomeadamente CNT oxidados com HNO_3 (CNT_HNO_3) e CNT *ball milling* (CNT_MB_M), bem como CNT magnéticos, impregnados com 2% de ferro (CNT@2%Fe, CNT@2%Fe_HNO_3 e CNT@2%Fe_MB_M), foram preparados. Os novos CM foram testados como RM na remoção biológica do Acid Orange 10 (AO10) com biomassa granular (GS) durante 29h de reação. Adicionalmente, CNT comerciais foram utilizados como RM na remoção biológica da ciprofloxacina (CIP) com GS, durante três ciclos de adição do antibiótico, de forma a compreender os mecanismos de remoção deste fármaco, sendo que nos primeiros dois ciclos a adsorção do composto aos CNT e à GS dificultam a interpretação.

A descoloração biológica do AO10, após 29h na presença de CM, mostraram melhorias comparativamente ao controlo sem CM (remoção de 29 ±3 %), por isso os materiais usados atuam como RM na reação biológica. Os melhores CM testados foram os CNT_MB_M, levando a 98±1 % de remoção de AO10 à velocidade de 2.94±0.18 d^{-1}. Ensaios abióticos não apresentam qualquer remoção do corante. Os resultados obtidos na presença de CM@2%Fe não apresentaram melhorias na velocidade de reação e também não permitem a degradação em condições abióticas, contradizendo resultados de estudos anteriores, onde foi verificado que o ferro também participa na transferência eletrónica, aumentando as velocidades de reação. A produção de metano não é afetada pela presença de diferentes CM.

Ensaios biológicos de remoção da CIP na presença de CNT (biotic.CNT.CIP), após três ciclos, revelaram 88±4 % de remoção do fármaco. A remoção em condições abióticas, apesar de muito menor, mostra a adsorção da CIP aos CNT (29±3 %) e na condição branco sem CNT (blank.CIP) foi apresentada uma remoção de 68±6 % devido à adsorção na GS. Os resultados obtidos evidenciam que a remoção da CIP ocorre por três mecanismos: adsorção à GS, adsorção aos CNT e redução biológica. Contudo, o efeito dos CNT como RM na redução da CIP não é evidente.

Palavras-chave: AO10; Ciprofloxacina; Biodegradação anaeróbia; Nanomaterias de carbono.

ABSTRACT

Wastewater pollution is one of the most serious problems worldwide. The rapid industrialization and significant rise in the population leads to the contamination of water resources with different micropollutants such as azo dyes and pharmaceuticals.

Current wastewater treatment technologies are not effective in the reduction of these compounds, resulting in the dissemination of them in soils, crops, surface water, groundwater and even in drinking water, leading to negative effects in public health and in aquatic life. Anaerobic treatments have been described for the biodegradation of micropollutants, however, the reactions proceed slowly due to the recalcitrant nature of the compounds, which is a limitation for the application of anaerobic bioprocesses. Therefore, adding to the reaction materials or compounds that act as redox mediator (RM), in order to accelerate the degradation reactions, it is intended to overcome that barrier. Some carbon materials (CM) have been described as good RM for the reduction of different micropollutants.

In this work, different carbon nanotubes (CNT) with modified surface chemistry, namely oxidized CNT with HNO_3 (CNT_HNO_3) and ball milling CNT (CNT_MB_M), as well as magnetic CNT, impregnated with 2% of iron (CNT@2%Fe; CNT@2%Fe_HNO_3 and CNT@2%Fe_MB_M), were prepared. The new CM were tested as RM in the biological Acid Orange 10 (AO10) removal with granular sludge (GS) over 29 h of reaction. In addition, commercial CNT were used as RM in ciprofloxacin (CIP) biological removal with GS, along three cycles of 24 h of the addition of the antibiotic, in order to understand the mechanisms of the removal of this pharmaceutical, since in the first two cycles the adsorption of the compound to the CNT and GS difficulted the interpretation.

Biologic decolourisation of AO10, after 29h in the presence of CM, shows improvements when compared with the control without CM (removal of 29 ±3 %), thus the materials used act as RM in the biologic reaction. The best CM tested was the CNT_MB_M, leading to 98±1 % of biological AO10 removal at the rate of 2.94±0.18 d^{-1}. Abiotic assays do not present any dye removal. The results obtained with the presence of CM@2%Fe do not showed improvement in the rate of the reaction and do not allows the degradation under abiotic conditions as well, which contradicts the results of a previous study, where iron was shown to also participate in the electron transfer, so improving the rates. The methane production was not affected by the presence of different CM.

Biological assay of CIP removal in the presence of CNT (biotic.CNT.CIP), after three cycles, showed 88±4 % of removal of the pharmaceutical. The removal in the abiotic conditions, although much less, show adsorption of CIP on CNT (29±3 %) and in the blank condition without CNT (blank.CIP) was showed 68±6 % removal due to adsorption on GS. The obtained results evidence that the removal of CIP occurs by three mechanisms: adsorption on GS, adsorption on CNT, and CIP biological reduction. However, the effect of CNT as RM on the reduction of CIP was not evident.

Keywords: AO10; Ciprofloxacin; Anaerobic biodegradation; Carbon nanomaterials.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACN – Acetonitrile

AD – Anaerobic Digestion

AO10 – Acid Orange 10

CIP – Ciprofloxacin

CM – Carbon Materials

CNT – Carbon Nanotube

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

CNT@2%Fe_HNO$_3$ – Composites of oxidized carbon nanotubes impregnated with 2% of iron

CNT@2%Fe_MB_M – Composites of ball milled carbon nanotubes impregnated with 2% of iron

CNT_HNO$_3$ – Oxidized Carbon Nanotubes

CNT_MB_M – Ball milled Carbon Nanotubes

COD – Carbon Oxygen Demand

DWCNT – Double-walled Carbon Nanotubes

Et – Ethanol

GC – Gas Chromatography

GS – Granular Sludge

HPLC – High Performance Liquid Chromatography

MNP – Magnetic Nanoparticles

MWCNT – Multi-walled Carbon Nanotubes

pH$_{pzc}$ – pH at the point of zero charge

RM – Redox Mediator

S$_{BET}$ – Specific surface area

S$_{meso}$ – Non-microporous surface area

VFA – Volatile Fatty Acid

V$_{micro}$ – Volume of microporous

V$_p$ – Volume of pores

VS – Volatile Solid

WWTP – Wastewater Treatment Plants

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1. INTRODUCTION

1.1 Environmental pollution

Environmental pollution is one of the concerning problems of today's world and comprises three types of pollution: air, water and soil. Water pollution, which may results from various sources of contaminants, has become one of the most serious problems worldwide, having impacts on ecosystems and human and animal life [2], [3].

Clean and potable water is one of the indispensable elements for all living organisms to survive. Yet, due to the rapid industrialization and significant rise in the population, the contamination of water resources appears worldwide. So, the demand for water has increased extremely, mainly in agriculture, industrial and domestic sectors, with consumption of 70, 22 and 8% of the available fresh water, respectively. This excessive consumption results in the generation of large amounts of wastewater containing many pollutants, due to industrial discharges, excess use of pesticides and fertilizers, pharmaceuticals residues and landfilling of domestic waters [2]–[4]. Some of the preoccupant classes of pollutants include dyes and pharmaceuticals [2], [5]. Their negative effects in public health and in aquatic life are related with their lethal effect, genotoxicity, mutagenicity and carcinogenicity, affecting also the ecosystems [2], [6]. Current wastewater treatment technologies such as Sorption, Coagulation-Floculation-Precipitation, Membrane Filtration, Advanced Oxidation Processes, etc., are not effective, resulting in the dissemination of these micropollutants in soils, crops, surface water, groundwater and even in drinking water [5]–[9]. So, it is urgent to develop effective processes to remove these pollutants from contaminated water or to degrade them totally or to non-toxic products [2].

1.1.1 Wastewater pollution from textile dying industries

Textile dying industries generate large amounts of contaminated effluents and receive great attention in the last decades. One of the reasons for that care is the high negative aesthetic visual impact of the colour that results from the release of dyes during dyeing and finishing processes and for their untreated or poorly treated discharges [4], [6], [10]. Indeed, during the dyeing process about 10-15% of the dyes used, especially azo dyes, do not bind on the textile fibers and are kept in water bath or released during washes after dyeing [6], [11].

Predominantly used in several industries, such as textile, food, paper, printing, leather, pharmaceutical and cosmetic, the azo dyes represent 50% of all the dyes used. Particularly, in the textile industry, up to 70% of dyes used are azo dyes[3], [4], [11], [12].

Azo dyes are aromatic and xenobiotic compounds with one or more azo linkage (-N=N-), which are recalcitrant in nature, as well as possessing carcinogenic properties[3], [4], [6], [10]–[15]. Usually, aromatic rings of azo dyes are substituted with sulfonic acid or other electro-withdrawing groups, which generate an electron deficiency and make the dye less susceptible to degradation by microorganisms [6], [10].However, these compounds have many consequences, being toxic towards aquatic life and mutagenic for humans [6].

The release of these effluents in the environment without the appropriated treatment, generates negative effects in aquatic environment [6], [10], [11]. Once in the aquatic courses, these coloured effluents cause reduction in sunlight penetration and solubility of gases, which, in turn, decrease photosynthetic activity and dissolved oxygen concentration, decreasing also the water quality. It causes also acute toxic effects on aquatic flora and fauna and still have an adverse impact in total organic carbon (TOC), biochemical oxygen demand (BOD) and chemical oxygen demand (COD) [3], [4], [6], [12]. In addition, dyes, even at lower concentrations exhibit high colour which will cause a negative aesthetic impact, as referred before [3], [4], [12].

Therefore, treatment of the effluents from textile industries should be not neglected and need to be further explored. Azo dyes resist to aerobic effluent treatment bioprocesses, but their removal can be obtained by conventional physical and chemical techniques such as, adsorption, coagulation-flocculation, oxidation and electrochemical methods. However, these methods have some disadvantages, such as being quite expensive, having operational problems, and generating huge quantities of sludge [10], [13], [16]–[18]. Thus, an economic alternative than physico-chemical methods with remarkable results, is the anaerobic and anaerobic/aerobic biological processes [13], [19], [20].

In the last years, some biological processes for the efficient removal of dyes have been developed, involving anaerobic biotransformations [13]. Most azo dyes are recalcitrant in the presence of oxygen in conventional techniques, because oxygen is a more efficient electron acceptor than azo dyes, but can be reduced under anaerobic conditions [11], [21]. Biological activated sludge is the most common processes and uses mixed cultures. However, high amounts of sludge are produced which is a disadvantage [9]. In anaerobic bioprocesses due to the slower grow of the microbial communities, much less biomass is produced. In addition, anaerobic bioprocesses need less space to be implemented, can treat wastewaters with higher COD concentrations, are less costly and convert the organic contaminants mainly into carbon

dioxide and methane (so, producing biogas). Still, the reduction of the azo dyes under anaerobic conditions proceeds very slowly, representing a limitation for the application of anaerobic processes for the treatment of dyeing wastewater. This drawback makes the application of high-rate anaerobic bioreactors difficult, because to reach a satisfactory extent of dyes reduction, long hydraulic retention times are required [21]. The application of redox mediator (RM) to accelerate the anaerobic bioprocesses, has been proved as an efficient way to speed the electron transfer from the oxidized substrate to the final electron acceptor, the dyes [22], [23]. This will be further discussed in section 1.2. It is worth to mention that under anaerobic conditions occurs the reduction of the azo dye linkages, yielding the corresponding aromatic amines. Although this reduction leads to decolourisation of the effluent, these amines need to be after removed by an aerobic treatment [14], [22]. So, the most effective biological strategy for the removal of azo dyes from wastewaters consist of a sequential anaerobic-aerobic treatment [24].

1.1.2 Wastewater pollution with pharmaceuticals

Used all around the world in hundreds of tonnes per year, pharmaceuticals are a large and diverse group of compounds crucial for the public health [8], [25]–[28], but due to the excessive use, these compounds have recently received more attention regarding their effect and behavior in the environment in special in water cycle [7].

The presence of pharmaceuticals active compounds in different aquatic mediums such as surface, drinking and wastewater is well documented [7], [28]–[30]. Almost of the urban wastewater are contaminated with medical compounds, that vary in different types and abundance [30]. In the last years, the discoveries of new illnesses and development of new drugs, the expansion of population and the inverting age structure in general population, justifies the increase of the use and consumption of pharmaceuticals. Pharmaceuticals have also been widely used in veterinary and in research studies [8], [25], [31].

The main source of pharmaceuticals are hospitals with high use of antibiotics that are not mostly fully metabolized in the body and then are excreted to the wastewater [27], [32]. Once inside the body, pharmaceuticals undergo several metabolic processes before they are excreted. However, not all these active compounds are completely metabolized by the organism and so are excreted in three forms: unmetabolized (completely intact), active metabolites (partially metabolized) or transformation products (completely metabolized) to sewage system and wastewater treatment plants (WTTP) [8], [26], [27], [30].

However, there are other sources, such as municipal sewage system, aquaculture, leaching from agriculture fields on spreading of manure as well as presence of livestock [27], [29] and also

pharmaceutical industries and research [1], [33], [34], as represented in figure 1. This is responsible for the widespread occurrence of these compounds in the water environment, mainly because the discharges of wastewater effluents [8], [25], [27], [28], [31].

The upgrading of analytic equipment's allows the detection of very low concentrations of several contaminants in different environmental mediums, with high accuracy [8]. At certain concentration, pharmaceuticals are toxic and may have adverse effects in ecosystems, affecting aquatic and terrestrial organisms, that may lead to development of bacterial resistance and antibiotic resistance genes in environment [7], [8], [25], [27], [31], [35].

As a consequence, many are found in Wastewater Treatment Plants (WWTP) [1]. Once entering WWTP, pharmaceuticals are not completely mineralized, due to its recalcitrant nature, even at low concentration. They are retained in the sludge or metabolized to a more hydrophilic but still persistent form, and can be found either adsorbed on the sludge and in the treated effluent [1], [7], [36]. Their removal has variable rates and depends on the properties of the substance, type of treatment and the capacity of the plant, and process conditions (e.g. sludge retention time, hydraulic retention time and temperature) [7], [32]. The low concentration of pharmaceuticals in wastewaters is one of the difficulties for their removal [36]. However, conventional WWTP are still widely used for wastewater treatment, mainly because they produce effluents with an acceptable quality at reasonable operating and maintenance costs [8]. The problem is that most of the studies on the fate of pharmaceuticals in WWTP focused only on aqueous phase, and concentrations of the pollutants in the sludge are rarely determined, but their screening show that these compounds are very present in this medium and most of the time, the decrease of pharmaceuticals in water treated in WWTP are mainly due to adsorption on sludge rather than properly being degraded [8]. This can represent a source of environmental contamination, because the agricultural reuse of sewage sludge is a common practice to improve the soil structure and provide nutrients [1]. Therefore, it is crucial to develop other processes for the treatment of pharmaceuticals in water and also those adsorbed on sludge. It is also essential the continuous monitoring of these compounds in water sources as well as toxicological studies [8], [28].

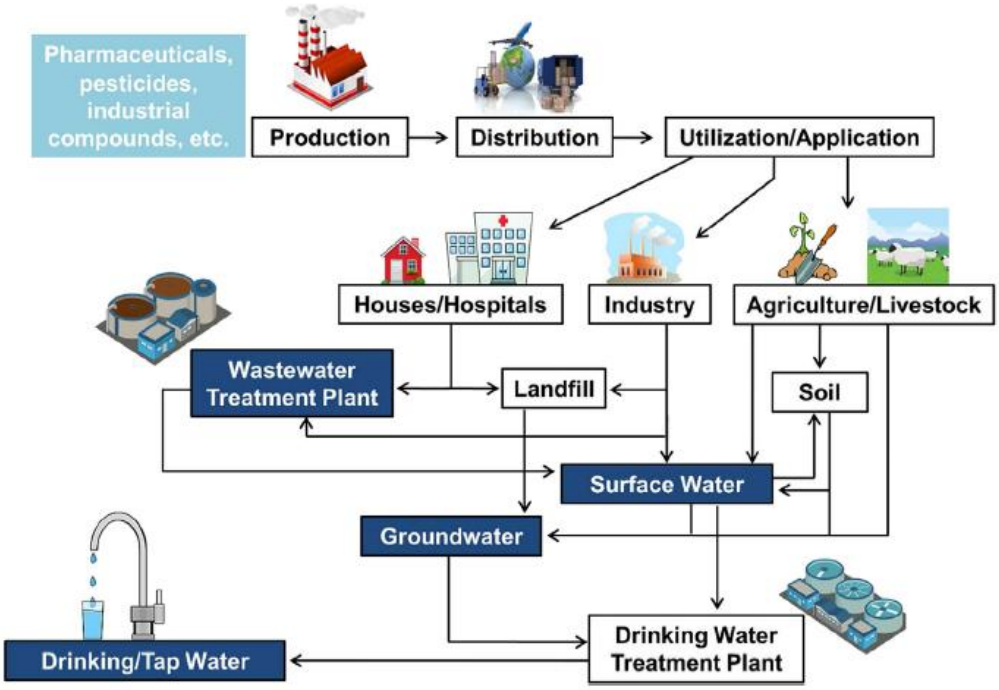

Figure 1. Representation of sources and distribution of micropollutants in the environment. Image reproduced from Barbosa *et al.* [1].

1.2 Anaerobic digestion

Anaerobic digestion (AD) is a complex food web, that sequentially degrades organic matter by a wide variety of microorganisms, in an anaerobic environment, with the production of methane. The processes involved can convert organic wastes and some anthropogenic pollutants (with strong electronegative groups, like azo dyes or nitroaromatic compounds) into stable and less harmful compounds, or that can be further degraded in a secondary aerobic treatment, as mentioned before for azo dyes. In addition, the biogas produced can be used as an alternative renewable energy source [37]–[39].

This multistep process of series and parallel reactions, proceeds in four successive stages, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis, where interactions between the syntrophic microorganisms species forms the anaerobic ecosystem [39]. The main groups of microorganisms that mediate these reactions are the fermentative bacteria, hydrogen-producing acetogenic bacteria, hydrogen-consuming acetogenic bacteria, carbon dioxide-reducing methanogens and aceticlastic methanogens [39].

Compared to typical aerobic treatment, this anaerobic process for wastewater treatment has some advantages, such as requiring low space, less nutrients and investment costs, as well as lower production of excess sludge, production of energy in the form of methane gas, high removal efficiencies for organic

pollutants, applicable at small and large scale, no or very little use of chemicals, and viable sludge can be stored under unfed conditions [39], [40].

However, the main disadvantage of this process is the slow rate [39]. Therefore, several studies have been made aiming to improve the rate of anaerobic biotransformations of recalcitrant compounds, by the use of RM [38]. These compounds favor the electron transfer, accepting electrons from chemical substances, or from the biological oxidation of a carbon source, and donating them to the pollutant of interest (azo dye or pharmaceutical) that acts as final electron acceptor, so RM act as intermediate electron transfer compounds [13], [21], [38]. Thus, their application may improve the efficiency of the start-up and operating of high-rate anaerobic reactors for wastewater treatment, especially when effluents have recalcitrant pollutants whose electron transfer rate can limit the overall process reaction [41].

1.2.1 Application of redox mediators in anaerobic biodegradation of pollutants

As mentioned above, the slow reduction of recalcitrant pollutants (like dyes and pharmaceuticals) limit the application of anaerobic wastewater treatments, which can be accelerated by the use of RM, adding them in the anaerobic environment [13], [21]. Extensively studied in last years, RM are compounds that can act as electron carriers in multiple redox reactions, increasing the reactions rate by one or more orders of magnitude, because they can be reversibly oxidized and reduced in the process [13], [22], [41]. Thus, to be an effective electron shuttle, RM should lower the reaction's activity energy. For this, its standard redox potential (E_0') should ideally be between the redox potentials of the electron donor and of the pollutant. In other words, the E_0' of RM may be higher or less negative than that of the electron donor, and at the same time, not much higher than that of the pollutant [22].

Many electron mediators such as quinone-like compounds, activated carbon (AC) and many other carbon materials (CM) and enzyme cofactors (for example, flavin adenine dinucleotide (FAD)) have been described [13], [14], [21], [22], [41]. Quinones are part of humic substances, constituting the electron accepting moieties of this compounds. Anthraquinone-2,6-disulfonate (AQDS) and antharaquinone-2-sulphonate (AQS), as model quinoid compounds, have received a greatest attention. Their use as RM lead to higher reductive efficiency in anaerobic bioreactors, operated at hydraulic retention time realistic for wastewater treatment practice. However, their application in anaerobic reactors is limited because of the need for continuous dosing, since they are soluble compounds, and this implies continuous expenses as well as continuous discharges of this additional chemical [21], [42].

In alternative, insoluble materials like AC, carbon nanotubes (CNT) and others CM have been shown to be viable RM [13], [14], [21], [23], [43]–[45]. The main advantages, compared with soluble RM, is

that they can be easily immobilized inside the reactors and do not need to be added continuously [46]. In addition, these insoluble RM can be easily removed from the treated solution by, for example, filtration [22]. At the same time, the possibility of modifying the chemical and/or physical properties of CM, in terms of surface area, pore size distribution, or by adding/removing chemical surface groups, in order to optimize their performance for specific applications, represents another advantage. Because they are retained in the reaction medium and being cyclically on oxidized and reduced forms, CM have been demonstrated as very effective electron shuttles at low concentrations, 0.1 g/L for instance [13], [14], [21].

1.3 Carbon materials

In the last years, CM have received worldwide attention and a unique place in nanoscience because of their excellent electrical, optical, thermal, chemical and mechanical properties that make them good materials for application in diverse areas (e.g. medical, electronic, electrochemical). The emergence of these materials over the past decade for use in a variety of innovative applications has reflected all their advantages[47], [48].

Currently, carbon is the fourth-most-abundant element in the world and the different arrangement of carbon atoms allows the formation of different allotropes, namely diamond, fullerenes, carbon nanoparticles, CNT, and graphene and its derivates [49]. Among them, CNT, particularly, have promoted high interest because of their nanosized, large scaffold, rich electronic states, excellent chemical stability, and high mechanical stability as will be described below[47], [49].

1.3.1 Carbon nanotubes

CNT were firstly described by Iijima in 1991 [50]. Since then, these nanomaterials have received a lot of attention because their unique and attractive properties, making them suitable materials for a wide variety of technology applications[48], [49], [51], [52].

CNT are an allotropic form of carbon, defined as cylindrical tubes made of graphene sheets rolled up, with a nanometer diameter and a length of several millimeters [2], [47], [51]–[55]. These nanomaterials are categorized into two structural forms, single-walled CNT (SWCNT), formed by one graphene sheet rolled up and multi-walled CNT (MWCNT), showing more than one concentric graphene sheets, as shown in figure 2. Usually, double-walled CNT (DWCNT) are also available when CNT are formed by two concentric graphene sheets, but this is a variant of MWCNT [47], [54], [56]. While SWCNT

are flexible, MWCNT are stiff rigid and rod-like structures [54]. The diameter of SWCNT and MWCNT can vary between 0.4 to 2 nm and 2 to 100 nm, depending on the synthesis conditions. Both materials can reach lengths of 0.2 to several millimeters [51], [52]. Another essential parameter that determines the structure and properties of CNT is the chirality (the angle between C-C bonds and the nanotubes axis). Three structures are possible, chiral, achiral zig-zag and armchair, that can be used to determine whether a particular SWCNT arrangement is semiconductive or metallic[51], [57]. Compared to SWCNT, MWCNT are more stable, more inert and less soluble in aqueous media, and their large-scale production is also relatively easier[51].

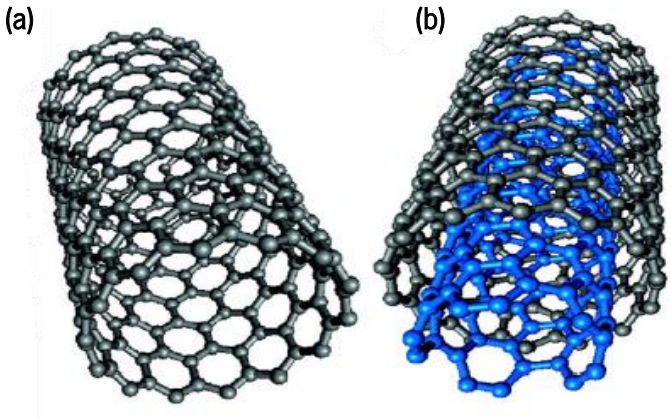

Figure 2. Structure representations of (a) SWCNT and (b)MWCNT. Image reproduced from Santhosh *et al.* [2].

The interest and application of CNT in many different fields is based on the exceptional structural, mechanical, electronic, electrochemical and optical properties which include: large surface area; ordered structure with controlled pore size distribution; small diameter; large scaffold; rich electronic states; high curvature; high thermal stability and conductivity; high electrical conductivity and light weight [2], [47], [49], [51]–[55].

All these properties make the CNT good materials for a wide range of applications in various areas such as medicine and biological engineering [47]–[49], [52], [53], [55]. Due to their high reactivity and adsorption capacity, their use in biotechnological and bioremediation processes, both as adsorbents and as catalysts has also been reported [13], [41], [46], [48], [58]. Moreover, these materials can be tailored for specific applications, as for example changing the surface functional groups, volume of pores, and combining different properties to prepare composite materials [51], [55], [58]–[61].

As mentioned above, CNT are good materials to prepare composites, for example the combination of CM with magnetic nanoparticles (MNP) allows the formation of magnetic carbon composites with

synergistic properties (catalytic and magnetic character) and advantages for some applications, such as the recovery of the composites by applying a magnetic field [54], [58]. The introduction of functional groups in CNT can also modifies their properties and improve their behavior for some applications, namely, the incorporation of heteroatoms (such as O,S,N,B) into the carbon surface modifies its electronic properties by introducing electron acceptors or donors and consequently the catalytic performance, which can enhance π bonding, leading to improved stability and electron transfer [60], [61]. For example, the catalytic performance of materials doping with N was improved in oxidation reactions [62]–[64], and in oxygen reduction reactions in proton-exchange fuel cells [65], [66].

In addition, CNT also provide fast electron transfer with excellent redox activity, making them good materials as RM [13], [41], [46], [49], [58].

Not withstand, the characteristics of the CNT, like the diameter, length, morphology, structure, chirality, quality and purity of the structure is strongly dependent on the method of preparation. Several techniques are described to produce CNT, namely Arc-discharge, Laser Ablation, Chemical Vapor Deposition (CVD) and Flame Synthesis [51], [53], [67]. One of the most widely used method is Arch-discharge [51].

1.3.2 Magnetic carbon nanocomposites

MNP, as compared with non-magnetic, have the advantage of rapid separation, due to its magnetic characteristic, but the disadvantage of being easily oxidized. In order to reduce this problem, they can be coated with different materials, improving their stability and introducing additional surface properties and functionalities [58], [68]. The materials used for the coating can be very different and the choice depends on the properties that are intended. CM are a versatile example of coating that can be used due to their chemical stability, biocompatibility and possibility of tailoring their textural and surface chemical properties [58].

The combination of MNP with CM allows the formation of magnetic carbon nanocomposites with synergistic properties. The conjugation of adsorptive and catalytic properties of both and magnetic character of MNP improves the perform of final material and renders it easier to be retained and recovered by applying a magnetic field [58].

1.4 Objectives

Various micropollutants are present in wastewaters, some of them being difficult to remove efficiently, so having negative effects in the environment as well as in public health. Among them are the azo dyes and pharmaceuticals.

Textile industries generate effluents with large amounts of synthetic dyes, which are released from the fibers during textile dying. Besides the impact of the visual aspect, many of the compounds existing in these effluents, namely azo dyes, are toxic and mutagenic, and very difficult to degrade. So, they are often discharged by the industries to municipal WWTP without treatment or poorly treated. In the WWTP these compounds are also not efficiently degraded and end, at some extent, in water bodies. Many researches have been done in an attempt to develop efficient processes for treating colored effluents. However, azo dyes are very recalcitrant and, in many cases, more toxic products are produced during decolourisation, namely aromatic amines. In addition, some physico-chemical processes introduce other chemicals, or only concentrate the pollutant, and are costly.

Used in large amounts around the world, pharmaceuticals are another group of pollutants present in aquatic environments. Mainly excreted in effluents from hospitals, but also from municipal sewage overflow, aquaculture, agriculture and pharmaceutical industries and research, these compounds, though appearing at low concentrations in nature, may cause adverse effects in ecosystems, affecting aquatic and terrestrial organisms, and leading to the development of bacterial antibiotic resistance. Like azo dyes, these compounds are recalcitrant even at low concentrations, and, in WWTP they can be found either in the treated effluent or adsorbed on the sludge.

In this work, the effect of different carbon nanomaterials as RM on the biological reduction of a model azo dye, Acid Orange 10 (AO10), and on the antibiotic ciprofloxacin (CIP), was studied. In the previous work in the group, a commercial CNT impregnated with 2% of Fe accelerated 79-fold the biological reduction of AO10. In this work, commercial CNT and CNT@2%Fe, but with different surface chemistry were prepared and the effect of the surface chemistry on the catalytic reduction of AO10, was assessed.

In a previous work of Antunes [36], testing CNT and CNT@2%Fe as RM on anaerobic biological removal of CIP, adsorption of CIP on biomass and on CM hampered the elucidation of the mechanisms of CIP removal. In this work, the objective was to understand the mechanism of CIP removal by analyzing the contributions of biological removal and adsorption on both biomass and CM. So, CNT were used as RM, but instead of one cycle as performed before, three cycles of CIP removal were evaluated, in order to saturate the CM, and the biomass.

2. MATERIALS AND METHODS

2.1 Chemicals and preparation of stock solutions

AO10 and CIP were purchased from Sigma-Aldrich, at the highest purity available (98%). A stock solution of AO10 of 25 mmol L^{-1} was prepared in deionized water. A stock solution of CIP at the concentration of 0.0151 mmol L^{-1} was also prepared in deionized water, but with addition of a few drops of hydrochloric acid (2M) under constant magnetic stirring, to facilitate the dissolution of CIP, due to its low solubility at neutral pH (water solubility of 30 g L^{-1} at 20°C, which is enhanced when it is in the ionic form).

Substrates and basal nutrients for the preparation of the medium for the bioreactors, were purchased from Sigma-Aldrich and used without further purification. A stock solution of Volatile Fatty Acids (VFA) containing acetate, propionate and butyrate in a COD based ratio of 1:10:10 was prepared in deionized water, and neutralized with NaOH, in the concentration of 100 g L^{-1} COD. A stock solution of ethanol was prepared with a concentration of 3 mol L^{-1}.

Acetonitrile (ACN) and formic acid (98%) for High Performance Liquid Chromatography (HPLC) analysis were purchased from Merk.

2.2 Carbon Materials: preparation and characterization

Preparation and characterization of carbon nanomaterials was made at the associated laboratory LSRE/LCM of Engineering Faculty of Porto University (FEUP).

A commercial MWCNT sample (Nanocyl 3100) was used as the starting material (CNT). According to the supplier, the CNT used have an average diameter of 9.5 nm, an average length of 1.5 μm and a carbon purity higher than 95%.

In order to produce a sample of CNT with a strongly acid character and large amount of surface groups, the original sample was oxidized with HNO_3 in the liquid phase (CNT_HNO_3), according to Gonçalves *et. al* 2010 [59]. Briefly, this oxidation was performed using a Pyrex round bottom flask containing 300 mL HNO_3 7 M and 4 g of CNT, connected to a condenser. The liquid was heated to boiling temperature with a heating mantle during 3 h. CNT were washed with distilled water to neutral pH, dried in an oven at 110 °C for 24 h and stored in a desiccator for later use [59].

N-doped CM are promising metal-free catalysts for a number of applications [60]. Aiming to obtain a CNT with N-groups incorporated (CNT_MB_M), 0.6 g of the commercial pristine CNT were mixed with

0.26 g of N using melamine as nitrogen precursors, and the mixture was ball milled in a closed flask without any gas flow in a Retsch MM200 equipment during 4 h at a constant vibration frequency of 15 vibrations/s [60]. Then, the resulting CNT were subjected to a thermal treatment under N_2 flow (100 cm^3 min^{-1}) until 600 °C and kept at this temperature during 1 h.

Commercial CNT were also used as catalysts and as support of the metal phase (Fe). For that, a material sample of 2%w of Fe monometallic catalyst supported on the CNT was prepared by incipient wetness impregnation from aqueous solution of the corresponding metal salt ($Fe(NO_3)_3$). After impregnation, the sample was dried at 100ºC for 24 h, heat treated under nitrogen flow at 400ºC for 1 h, and finally reduced at 400ºC in hydrogen flow for 3 h (CNT@2%Fe) [58]. The modified surface materials CNT_HNO_3 and CNT_MB_M were also impregnated with iron, forming the samples CNT@2%Fe_HNO_3 and CNT@2%Fe_MB_M.

The textural and chemical properties of the materials were characterized by N_2 adsorption at -196ºC, pore size distribution, pH at the point of zero-charge (pH_{pzc}) and elemental analysis, as described in previous studies [58]–[60].

2.3 Biodegradation of azo dye Acid Orange 10

The prepared materials, CNT, CNT_HNO_3, CNT_MB_M, CNT@2%Fe, CNT@2%Fe_HNO_3 and CNT@2%Fe_MB_M were tested as RM on azo dye reduction, using AO10 as model compound. For evaluation of new materials, dyes are excellent model compounds, because they are observable to naked eye and their removal can be easily followed by spectrophotometry. Also, they exhibit higher colour even at low concentrations, due to high extinction molar coefficients.

Biological reduction of AO10 was conducted in 70 mL serum bottles, sealed with a butyl rubber stopper, containing 25 mL of buffered medium at a pH of 7 with $NaHCO_3$ (2.5 g L^{-1}). Basal nutrients were: NH_4Cl (2.8 g L^{-1}), $CaCl_2$ (0.06 g L^{-1}), KH_2PO_4 (2.5 g L^{-1}), $MgSO_4.7H_2O$ (1.0 g L^{-1}). As primary electron donor substrate, VFA mixture was added at the medium. Granular sludge (GS), collected from an anaerobic internal circulation reactor of a brewery wastewater treatment plant, was the inoculum at a concentration of 2 g L^{-1} of volatile solids (VS). CM were present at the concentration of 0.1 g L^{-1}, based on previous studies [13], [58]. The medium was flushed with N_2/CO_2 (80%/20%) and incubated overnight at 37ºC in a rotary shaker at 105 rpm, in order to promote the consumption of residual substrate. After the pre-incubation period, the bioreactors were flushed again with N_2/CO_2 (80%/20%) and AO10 and VFA were added from the stock solution to the desired concentration: 0.5 mmol L^{-1} and 2 g L^{-1} of COD, respectively.

Controls include: blank assays without substrate in the presence and absence of CM, biological assays without CM and abiotic assays in the presence of materials. All experiments were prepared in triplicate.

2.3.1 Analytical techniques

Samples were withdrawn from the bioreactors at increasing times along the reaction, centrifuged for 10 min at 15.000 rpm, and diluted up to an absorbance of less than 1, with a freshly solution of ascorbic acid (200 mg L^{-1}) to prevent aromatic amines oxidation. AO10 decolourisation was followed by spectrophotometry, measuring the absorbance at the dye wavelength of maximum absorbance, 480 nm, in a 96-well plate reader (Biotek® Synergy HT, Gen5 Data Analysis Software). AO10 concentration was calculated with the molar extinction coefficient of the dye (ε_{480nm}= 22.27 mmol L^{-1} cm^{-1}). A calibration curve was made by analyzing AO10 solutions at increasing concentrations in order to determine molar extinction coefficient (annex I). The percentage of azo dye removal was calculated according to equation:

$$Removal\ (\%) = 100 - \left({}^{C_t}/_{C_0}\right) \times 100 \quad (1)$$

Where, C_t is AO10 concentration at the time t and C_0 is the initial concentration.

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

$$C_t = C_i + C_0\, e^{-t/k} \quad (2)$$

Where C_t and C_0 are the concentrations at time t and initial; C_i is the offset, a value closed to the asymptotic of the Y variable (C) for larger time (t) values, and k is the first order rate constant (d^{-1}).

The concentration of CH_4 present in the biogas produced in each bottle was determined by Gas chromatography (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 220ºC. Nitrogen was the carrier gas at a flow rate of 30 mL min^{-1}. 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, a standard CH_4 was injected (with 40% CH_4) and, afterwards the samples were also injected.

2.4 Biodegradation of ciprofloxacin

Pristine CNT were tested as RM on the anaerobic biodegradation of CIP. Biological reductions were conducted in 200 mL serum bottles, containing 100 mL of work volume, composed by the medium, the inoculum, the substrate, CIP and CNT (0.1 g L^{-1}). Anaerobic basic medium used was prepared as described by Angelidaki and Sanders [69]. Ethanol (Et) was the primary electron donating substrate at the concentration of 30 mmol L^{-1}. GS was used as inoculum at a concentration of 3.0 g L^{-1} VS. CIP was added at the concentration of 0.0151 mmol L^{-1}. Three cycles of 24h of CIP addition were evaluated. Conditions tested include blank controls with and without materials, biological controls in the absence of CNT and/or CIP, and abiotic controls (Table 1).

Table 1. Discrimination of the compounds included in each sample tested. GS – Granular sludge; Et – Ethanol; CNT – Carbon nanotubes; CIP – Ciprofloxacin.

Sample name	Sample constitution
Blank.CIP	GS + CIP
Blank.CNT.CIP	GS + CNT + CIP
Biotic	GS + Et
Biotic.CIP	GS + Et + CIP
Biotic.CNT	GS + Et + CNT
Biotic.CNT.CIP	GS + Et + CNT + CIP
Abiotic.CNT.CIP	Et + CNT + CIP

Similarly, as the described for AO10, the bioreactors were flushed with N_2/CO_2 (80:20 % v/v) and incubated overnight at 37ºC in a rotary shaker at 105 rpm, in order to consume all the residual substrate. Thereafter the CIP and ethanol were added from the stock solution at the desired concentration and both were monitored during 24 hours (each cycle) by HPLC.

2.4.1 Analytical techniques

Samples were withdrawn from the bioreactors at different reaction times, centrifuged for 10 min at 15.000 rpm to remove the GS and CNT, and the supernatants were filtered with Spartan 13/0.2 RC filters, Whatman 0.2 μm pore size. The concentration of CIP was followed by HPLC analyses in an Ultra HPLC (Shimadzu Nexera XZ) equipped with a diode array detector (SPD-M20A), autosampler (SIL-30AC),

degassing unit (DGU-20A5R), LC -30AD solvent delivery unit, and a 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: 0.1 % formic acid aqueous solution and ACN. The compounds were eluted at a flow rate of 0.8 mL min^{-1} and at 40°C, with an increase from 5 to 15% of ACN over 6 minutes, followed by an isocratic step during 12 minutes, then from 15 to 40% of ACN during 12 minutes, condition maintained for 10 minutes [70]. CIP was monitored at 275 nm and its retention time (Rt) was 13.2 min. A calibration curve was made by analyzing CIP solutions at increasing concentrations ($C = 65.76x - 1587$; $r^2 = 0.997$) (annex II). The percentage of pharmaceuticals removal was calculated according to equation 1. First order reduction rate constants were calculated in OriginPro software, applying the equation 2.

The consumption of the substrate (ethanol) 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 was 60°C, and the elution flow rate was 0.7 mL min^{-1}. The mobile phase was a solution of sulfuric acid (5 mM). For the analysis, a volume of 2 mL was collected from the reactors at different reaction times. Samples were centrifuged at 15000 rpm during 10 min and filtered with Spartan 13/0.2 RC filters, Whatman 0.2 μm pore size [71], [72].

The amount of CH_4 produced was determined as described previously for the experiment with AO10.

3. RESULTS AND DISCUSSION

3.1 Characterization of carbon materials

The CM tested as RM in this work were characterized in terms of pH_{pzc}, specific surface area (S_{BET}), non-microporous surface area (S_{meso}), Volume of pores (V_p) and volume of microporous (V_{micro}) (Table 2). Figure 3 presents a summarized representation of the modifications in CNT with the different treatments applied, and the respective values of pH_{pzc}, S_{BET} and V_p. The pH_{pzc} values are related with the surface groups in the materials, so since the treatments performed on CM caused changes in the surface chemistry, changes on the pH_{pzc} values are also expected.

Table 2. pH_{pzc} and textural characterization of CM tested as RM in the assays for the removal of AO10 and CIP removal

Samples	pH_{pzc} (±0.2)	S_{BET} (m^2/g)	S_{meso} (m^2/g)	V_p (cm^3/g)	V_{micro} (cm^3/g)
CNT	6.6	201	201	0.416	0.000
CNT_HNO_3	2.2	223	223	0.448	0.000
CNT_MB_M	6.7	225	225	0.503	0.000
CNT@2%Fe	6.6	196	196	0.440	0.000
CNT@2%Fe_HNO_3	2.2	208	208	0.444	0.000
CNT@2%Fe_MB_M	6.7	243	243	0.581	0.000

pH_{pzc} – pH at the point of zero charge; S_{BET} – specific surface area; S_{meso} – non-microporous surface area; V_p – volume of pores; V_{micro} – volume of microporous

The treatment with nitric acid strongly acidifies the original CNT, which is due to the incorporation of a large amount of oxygen containing groups, leading to a decrease of the pH_{pzc} of the original material, from 6.6 to 2.2 (sample CNT_HNO_3) (Table 2). Concerning the CM prepared by ball milled, with also incorporation of N-groups, the thermal treatment applied, removes the groups at the surface of the CNT, Therefore, as a result, basic samples are expected, as observed previously for activated carbon [21]. However, CNT_MB_M had similar pH_{pzc} of the original CNT, which may be related with the fact that original CNT have already low amount of surface groups. The incorporation of Fe did not cause changes on pH_{pzc}, so samples CNT@2%Fe_MB_M and CNT@2%Fe_HNO_3 have a pH_{pzc} similar to the materials before incorporation of iron.

Although the differences on the S_{BET} values between the different CM are not higher, the functionalization of CNT lead to a slight increase of S_{BET}, from 201 $m^2 g^{-1}$ (original CNT) to 223 $m^2 g^{-1}$, for sample CNT_HNO_3, and to 225 $m^2 g^{-1}$ for sample CNT_MB_M. These differences suggest that during the treatments some changes in the CNT structure could occur. In oxidized materials, this increase may be explained by the fact that the oxidative process leads to the open up the endcaps of CNT and create sidewall openings [59]. Moreover, the oxidizing agent (HNO_3) may cause the removal of amorphous carbon that can be in CNT [73]. The S_{BET} increase observed for samples submitted to ball milling process can be due to the first stage under ball milling (in the absence of any N-precursor) that reduces the entanglement of the CNT, leading to shorter CNT by breaking up the tubes without affecting the tube diameters [60]. However, the incorporation of 2% of iron caused a slight decrease of the surface area, in CNT@2%Fe and CNT@2%Fe_HNO_3 samples, probably due to the iron impregnated that occupies the spaces, which may block the access of N_2 to the inner cavities during the process of characterization by N_2 adsorption isotherms at -196°C . Contrary, in the CNT@2%Fe_MB_M, a higher S_{BET} is verified, which may be due to the defects caused in CNT structure due to N-groups incorporation (Figure 3). Since CNT do not have microporous, the values of S_{meso} and S_{BET} are similar.

In addition to the changes observed in the S_{BET}, some differences were detected in the pore volumes determined from the N_2 uptakes. The V_p increases for all modified CNT compared to the commercial CNT. Most of the pore volume results from the free space in the CNT bundles, that is why the V_{micro} is zero for all the CM. The increase of V_p in the oxidized CNT, may be to the collapse of the pores during oxidizing treatment. In the case of functionalized CNT by ball milling, and introduction of nitrogen functionalities, a decrease of the pore volume would be expected, since ball milling leads to a higher agglomeration of the material, reducing the space between the tubes, while the introduction of N-groups on the CNT surface may favor the interaction between the tubes, resulting in a higher level of agglomeration of the material [61]. However, the opposite was observed for the CNT_MB_M prepared in this study, which may be due to the occurrence of defects on the CNT structure during the first step, which involves a mechanical treatment, promoting the breaking of the tubes. The thermal treatment at 600°C, and incorporation of N-groups may also contribute for these defects. It is worth to note that the increase of S_{BET} of the functionalized CNT, may also be related with the increase of V_p.

After the incorporation of Fe sample prepared by ball milling, sample CNT@2%Fe_MB_M, presented higher S_{BET} and V_p, which may be caused by an additional subjection of the materials to high temperatures. However, the same was not observed for sample CNT@2%Fe_HNO_3, presumably due to protection by surface groups.

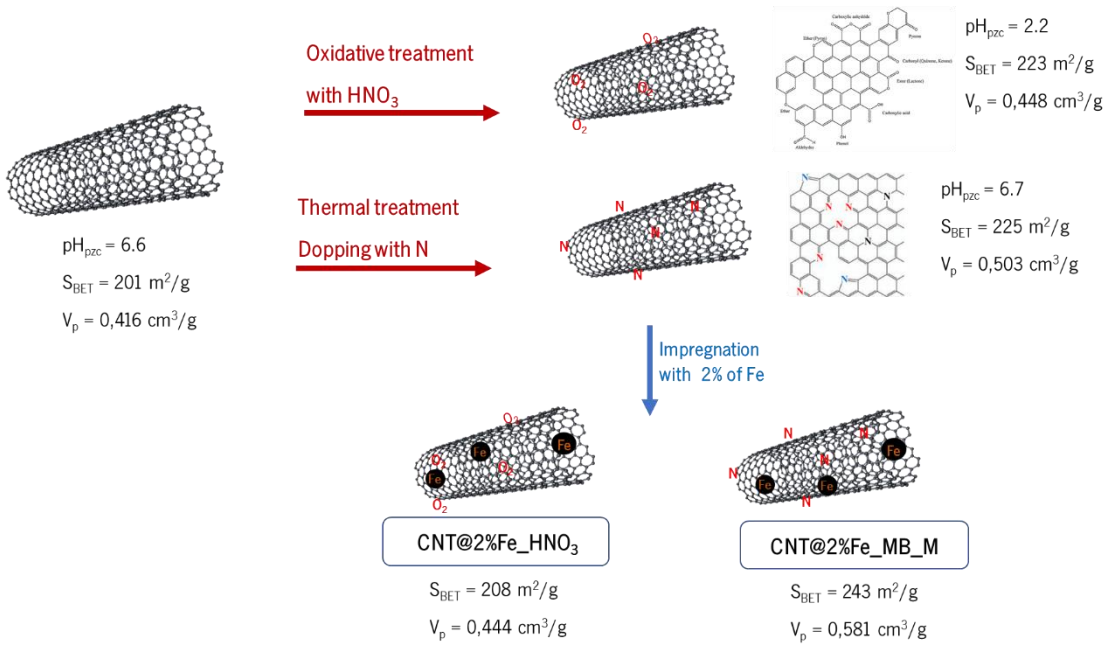

Figure 3. Representation of the modified CNT, and respective values of pH_{pzc}, S_{BET} and V_p of each material.

As can be seen by the results of elemental analysis, original CNT are mainly composed of carbon as expected (Table 3), with a very low percentage of hydrogen and oxygen. Yet low amount of oxygen reflects the presence of very low amounts, or absence, of oxygen reach groups. CNT_HNO_3 and CNT_MB_M are also mainly composed by carbon. The percentage of nitrogen in the sample prepared by ball-milling with N-groups incorporation is 1,69, showing that incorporation was successfully. Sample prepared by oxidation has no N, but has higher amount of oxygen (≈ 3-fold higher), which is due to the incorporation of oxygen rich groups.

Table 3. Elemental analysis of the CM used in the assays of AO10 and CIP removal

Sample	N (%)	C (%)	H (%)	S (%)	O (%)	Sum (%)
CNT	0.00	100.4	0.11	0.00	0.06	100.5
CNT_HNO_3	0.00	98.0	0.19	0.15	1.25	99.6
CNT_MB_M	1.69	96.4	0.18	0.00	0.39	98.6

3.2 Biodegradation of azo dye Acid Orange 10

Removal of AO10 under biologic and abiotic conditions was followed over 29 h, until it reached the equilibrium and no more decolourisation was observed. Figure 4 presents the decrease in the concentration of AO10 along the time. It can be observed that the reaction followed first-order kinetics. The removal percentage and the rate (d^{-1}) of the decolourisation were calculated at different conditions and are given in table 4. The removal of AO10 in the control assay without CM (only GS and substrate) was 29±3 %, evidencing that this compound is recalcitrant. The application of all CM used, improved significantly the removal and the rate of reaction, as can be observed in figure 4. So, CM behave as RM, increasing the electron transfer between the substrate and the final acceptor (azo dye). The presence of different CNT in abiotic assays do not caused any removal of AO10, indicating that there is no adsorption on the materials, at least in significant quantity, neither transformation of the dye due to the medium composition and tested conditions. It is important to note that, although CM are good adsorbers due to the high specific surface area, the amount used in this study was very low, only 0.1 g L^{-1}. The low amount of CM required to act as RM is based on the fact that there change between the reduced to the oxidized state during the process of electron transfer. On the other hand, blank assays with CM and GS, but without substrate, present removals of 43±1, 41±3 and 39±4 %, when with CNT, CNT_HNO_3 and CNT_MB_M, respectively. These results suggest that CM can stimulate the microorganisms and provide faster electron transfer than in the assay without CM, even without substrate addition, probably because of the presence of some residual substrate (which act as electron donor) that was not consumed during pre-incubation. The biggest removals were verified in biological assays, with substrate, in the presence of different CM. In these conditions, almost all of the decolourisation was reached over 29 h, with 97±1 % of removal in the experiments with CNT, 94±1 % with CNT_HNO_3, and 98±1 % with CNT_MB_M, occurring at reduction rates of 2.64±0.15, 2.32±0.14 and 2.94±0.18 d^{-1} for assays with CNT, CNT_HNO_3 and CNT_MB_M, respectively. Relatively to commercial CNT, CNT_MB_M, although the similar percentage of dye removal, have slightly improved the reduction rate, while with CNT_HNO_3 the extent of decolourisation and also in the reaction rate where lower, as compared with the other two CM.

Due to their amphoteric character, CM may have positively or negatively charged surfaces, depending on the pH of the solution and on their pH_{pzc}. So, the surface of materials becomes positively charged at $pH < pH_{pzc}$ and negatively charged at $pH > pH_{pzc}$ [58]. Concerning the azo dye used in this study, it is anionic, thus negatively charged when in solution. Therefore, adsorption and electron transfer are more favorable when the material's surface is positively charged in solution (opposite charge of that of the dye), while electrostatic repulsion occurs between negatively charged CM and the anionic dye,

making the adsorption and electron transfer harder. CNT and CNT_MB_M present similar pH_{pzc}, close to the neutrality, while a high difference is verified in CNT_HNO_3, being an acidic CM, pH_{pzc} of 2.2. At the pH of the medium (circa 7), CNT_HNO_3, are negatively charged when in the solution medium, favoring the repulsion of the AO10 and, so, hampering the reaction and the adsorption. Besides that, CNT_HNO_3 also has a high content of surface electron-withdrawing oxygenated groups, which make the surface access difficult for the dye, as well as difficult the electron transfer from the material to dye, making it worse material as electron shuttle [59]. CNT_MB_M and CNT, present similar pH_{pzc} and, indeed, similar behavior in the decolourisation reaction was observed, with closed extent of decolourisation, but a slight increase in the rate of the reaction for modified CNT. So, this improvement with CNT_MB_M may be due to the increase of S_{BET} and V_p, that leads to a better access of the dye and, consequently, favoring the approximation of the dye to CM and, so, facilitating the reduction of it. Moreover, the nitrogen atoms in CNT_MB_M provide additional electrons to the material which can improve the catalytic activity and present a higher reaction rate [60].

A previous study of AO10 decolourisation (1 mM), with the use of CNT (0.1 g L^{-1}) as RM, presented a removal of 98±2 %, at the rate of 3.16±0.65 d^{-1} [13], which is in agreement with the results obtained in this work, although a lower amount of AO10 used (0.5 mM) and the surface of those CNT was higher (about 1.6-fold higher).

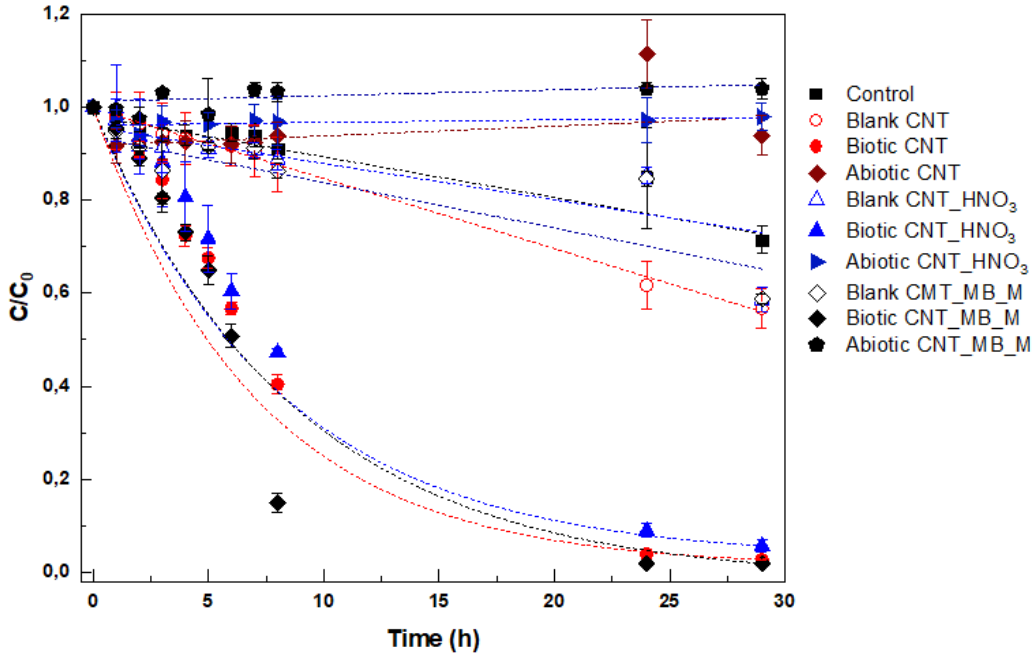

Figure 4. Decolourisation of AO10 using CNT, CNT_MB_M and CNT_HNO_3 as RM. The results include the control assay in the absence of CM; the blank assays with the CM and GS, but without substrate; the biotic assays with CM, GS and substrate; and the abiotic assays with CM in the absence of GS.

The modified CNT, but impregnated with 2% of iron, CNT@2%Fe, CNT@2%Fe_HNO_3 and CNT@2%Fe_MB_M, were also tested as RM for AO10 decolourisation. The removals have also followed a first order kinetic and the decrease in AO10 concentration along the time is present in figure 5. The percentage of removal and the rate of reaction (d^{-1}) are presented in table 4. In these assays, CM@2%Fe showed similar behavior as compared with similar CM but without 2% of Fe. Any removal of AO10 was obtained in abiotic assays and, in the blanks, similarly as the obtained with the materials without iron, a removal of 43±3, 45±3 and 49±2% was obtained with CNT@2%Fe, CNT@2%Fe_HNO_3 and CNT@2%Fe_MB_M, respectively. In the biologic assays in the presence of these materials, removals of AO10 were: 98±1 % for CNT@2%Fe and CNT@2%Fe_MB_M, and of 93±1 % for CNT@2%Fe_HNO_3. The removal rates for these assays were 2.38±0.39 d^{-1} (CNT@2%Fe), 1.58±0.23 d^{-1} (CNT@2%Fe_HNO_3) and 2.41±0.18 d^{-1} (CNT@2%Fe_MB_M). Like for modified CNT, the presence of all CM@2%Fe shows improvement in AO10 removal compared with control assay without CM (present 29±3 % removal), acting as RM for AO10 transformation. CNT@2%Fe shows similar behavior in AO10 removal as CNT@2%Fe_MB_M, suggesting that does not have significative differences between these materials that influence the AO10 transformation. In other hand, CNT@2%Fe_HNO_3 was worse as RM in AO10 decolourisation, and smaller percentage of removal and rate of the reaction, as compared with two other CM@2%Fe, was obtained in that assay. These results can be explained by the repulsive effect of this material to the dyes, as explained above, due to its pH_{pzc}, and by the difficult access to the surface of the material due to the presence of big functional groups. The presence of iron slightly worsened the rates of reaction, probably due to the less access of the dye. This result contradicts previous results, where iron was shown to also participate in the electron transfer, so improving the rates.

Indeed, according to a mechanism for AO10 reduction suggested by Pereira et al. 2017 [58], in the presence of CNT@2%Fe the electron transfer may occur through three possible pathways: the biological oxidation of the co-substrate (VFA) to the final acceptor, AO10; the biological oxidation of co-substrate to the CNT of the composite and then to the final acceptor, AO10; or from Fe (Fe^{2+}) impregnated in CNT to the carbon of the composite and then to the final acceptor, AO10. In that previous study, 98±3 % of AO10 removal was obtained, at the rate of 16.66±2.00 d^{-1}, in biotic assay, and a removal of 92±1 %, at the rate of 13.09±1.10 d^{-1}, in abiotic assay, which is in disagreement with the result obtained in this work. However, the amount of CM was 5 times higher (0.5 g L^{-1}). Notwithstanding, the results obtained in present work (with CM@2%Fe) does not show any improvement in the reaction rate of biotic assays when compared with biotic assays with original CNT, as well as does not present any dye removal in abiotic assay. These justification for these unexpected results is still being researched, however a possible

explanation may be the different commercial CNT used as matrix for the preparation of the new CM, which, although the same reference, came from a different lot, which may have slight differences in surface characteristics [74]–[76]. In terms of synthesis, CNT@2%Fe used in this work were produced in similar way as in previous work, but from commercial CNT from another lot, which may have introduced some differences in the CNT@2%Fe produced, which in turn may have affected their performance in this work. However, the fact that these CM are magnetic is an advantage since they can be removed easily from the solution after the reaction, by a magnetic field, and reused for another reactions [58].

Based on similar studies [58], we can suggest that the removal of AO10 from the reaction medium was due to the reduction of the dye, with the consequently formation of aromatic amines. This can be proved by HPLC analyses, like described in other works [13], [41], [58], however it was not possible to perform in this work, due to the unavailability of the equipment during the period of the work.

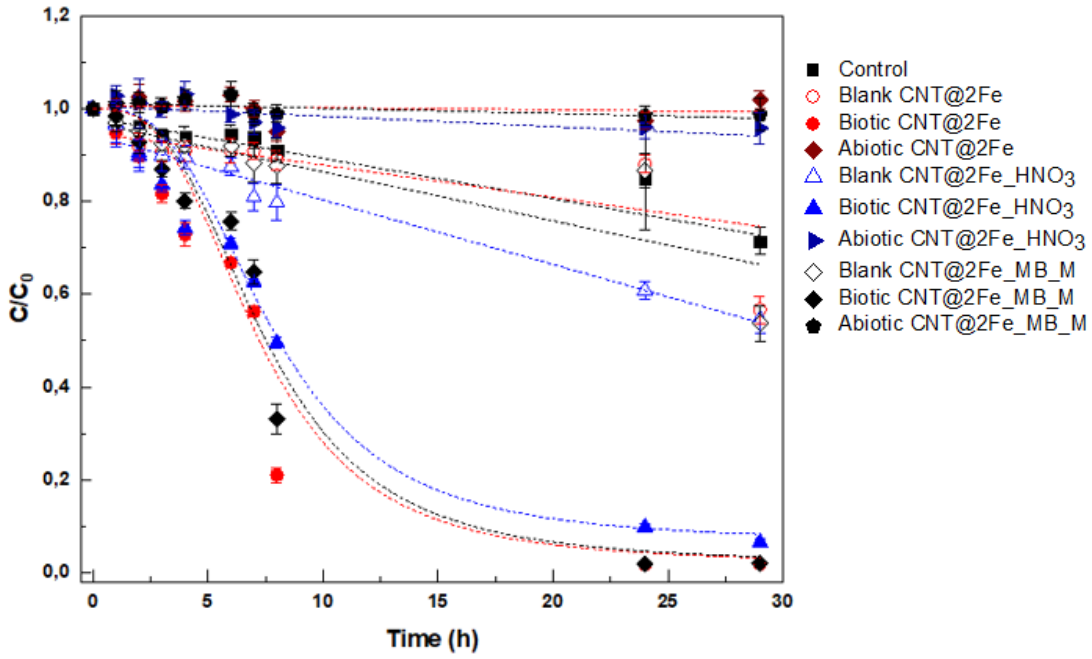

Figure 5. Biological reduction of AO10 using the materials CNT@2%Fe, CNT@2%Fe _MB_M and CNT@2%Fe _HNO_3 as RM. The results include control assay in the absence of CM; blank assays with the respective CM and GS, but without substrate; biotic assays with CM, GS and substrate; and abiotic assays with CM in the absence of GS.

Table 4. Removal (%) and rate (d^{-1}) of decolourisation of the AO10 solution, with 0.1 g L^{-1} of CM. The results include the control assay in the absence of CM; the blank assays with the respective CM and GS, but without substrate; the biotic assays with CM, GS and substrate; and the abiotic assays with CM in the absence of GS. The values of the concentration of methane (mmol L^{-1}) after 29 h of biological treatment of AO10 are also presented. GS – Granular sludge; VFA – Volatile Fatty Acid; CM – Carbon material; AO10 – Acid Orange 10

Sample name	Sample constitution	Removal (%)	Rate (d^{-1})	CH_4 (mmol L^{-1})
Control	GS + VFA + AO10	29 ± 3	0.27 ± 0.03	12.01 ± 0.56
Blank CNT	GS + CM + AO10	43 ± 1	0.37 ± 0.01	0
Biotic CNT	GS + VFA + CM + AO10	97 ± 1	2.64 ± 0.15	11.88 ± 0.33
Abiotic CNT	VFA + CM + AO10	0	n.a.	n.a.
Blank CNT_HNO_3	GS + CM + AO10	41 ± 3	0.39 ± 0.01	0
Biotic CNT_HNO_3	GS + VFA + CM + AO10	94 ± 1	2.32 ± 0.14	11.82 ± 0.40
Abiotic CNT_HNO_3	VFA + CM + AO10	0	n.a.	n.a.
Blank CNT_MB_M	GS + CM + AO10	39 ± 4	0.38 ± 0.01	0
Biotic CNT_MB_M	GS + VFA + CM + AO10	98 ± 1	2.94 ± 0.18	12.35 ± 0.29
Abiotic CNT_MB_M	VFA + CM + AO10	0	n.a.	n.a.
Blank CNT@2Fe	GS + CM + AO10	43 ± 3	0.32 ± 0.03	0
Biotic CNT@2Fe	GS + VFA + CM + AO10	98 ± 1	2.38 ± 0.39	13.14 ± 0.01
Abiotic CNT@2Fe	VFA + CM + AO10	0	n.a.	n.a.
Blank CNT@2Fe_HNO_3	GS + CM + AO10	45 ± 3	0.34 ± 0.04	0
Biotic CNT@2Fe_HNO_3	GS + VFA + CM + AO10	93 ± 1	1.58 ± 0.23	10.49 ± 0.46
Abiotic CNT@2Fe_HNO_3	VFA + CM + AO10	0	n.a.	n.a.
Blank CNT@2Fe_MB_M	GS + CM + AO10	49 ± 2	0.37 ± 0.03	0
Biotic CNT@2Fe_MB_M	GS + VFA + CM + AO10	98 ± 1	2.41 ± 0.18	12.65 ± 0.37
Abiotic CNT@2Fe_MB_M	VFA + CM + AO10	0	n.a.	n.a.

n.a. – not applicable.

The methane produced in the biologic decolourisation of AO10 are similar in all conditions, showing that the microorganisms are active and have similar behavior in the use of the substrate, VFA. The amount of CM used in this assay is small and in a short reaction period, so they do not have effect in the methane production, neither stimulates nor inhibits, contrary to other reported studies [77]–[79], where the reaction period is of several days and the concentration of CM used is higher in most studies (0.1 to 5 g L^{-1}). The use of mixed cultures in this assay, contrary to the use of pure cultures in other reported works [79] can also make the difference in the variation of methane production.

3.3 Removal of the pharmaceutical ciprofloxacin

The removal of CIP under biotic and abiotic conditions was followed by HPLC over 3 cycles of 24h. An exemplar HPLC chromatogram are presented in annex III, where is visible the reduction of the peak of CIP. As shown in figure 6, the reaction followed a first order kinetics. The percentage of removal and rate of reaction (d^{-1}) were calculated and are presented in table 5. In the cycle 1, the removal of CIP is 90 % in the blank without CNT, showing the high adsorption of the compound on biomass. In the blank with CNT, the 94 % of removal are due to adsorption on the CM and on biomass. For the other conditions, although a small increase in CIP removal, is difficult to discriminate between adsorption and biological reduction of the pharmaceutical compound. In the cycle 2, more differences between conditions are visible. In the abiotic control, the removal of pharmaceutical was 80±8 %, at the rate of 7.2±3.1 d^{-1}, showing greatest difficulties in adsorption of CIP on CM compared with cycle 1 (20.6±18.9 d^{-1}). Pharmaceutical removal decreases to 79±2 % in blank assay (blank.CIP), and 84±3 % in blank with CNT (blank.CIP.CNT), showing less adsorption of CIP on biomass and on CM than in cycle 1, because the presence of the adsorbed pharmaceutical from the previous cycle. In the biologic assays a small decrease in CIP removal, compared with cycle 1, is already visible, but the differences are not yet significant to discriminate the removal mechanism. In the cycle 3, the condition with more differences comparing to previous cycles, is the abiotic control with CNT (abiotic.CNT.CIP), with a decrease of CIP removal to 29±3 %, showing the saturation of the CM. In the blank assays, the removal of CIP was 68±6 % in the absence of CNT (blank.CIP), and 78±1 % with CNT (blank.CNT.CIP). Although the smaller pharmaceutical removal than in previous cycles, the differences are not as high as in abiotic assay, showing that the saturation of CNT is reached faster than the saturation of biomass, because the biomass have more surface area available for adsorption, than CNT. For the biotic assay without CNT (biotic.CIP), pharmaceutical removal at the end of 3rd cycle was 86±2 % at the rate of 33.9±3.5 d^{-1}, and in the presence of CNT, was 88±4 % at the rate of 36.1±5.3 d^{-1} In these conditions the pharmaceutical removal remains high, contrary to what was observed in other conditions of this cycle, suggesting that besides adsorption biological reduction of the pharmaceutical also occurs.

After three cycles of the saturation of the sludge and the CM, the obtained results suggest the occurrence of different mechanisms of CIP removal: adsorption on sludge and/or on CM, and biological reduction. Adsorption on biomass was expected based on previous studies reporting that removal of CIP is mainly due to adsorption on activated sludge rather to biodegradation, driven by hydrophobic and electrostatic interactions, as well as size exclusion [36], [80]–[83]. In the biological assay without CM, two mechanisms may be present, adsorption of CIP on GS and its reduction due to electrons generated

by the oxidation of ethanol. The occurrence of these two mechanisms possibly explain the higher percentage of CIP removal in biotic.CIP assay compared with the assay without substrate (blank.CIP assay), where only adsorption to GS occurs. When CM are present, adsorption of CIP on GS and on CM is possible, in the blank.CNT.CIP assay, which may justify the higher CIP removal when compared to blank.CIP assay. In addition, three mechanisms can occur in biotic.CNT.CIP assay, adsorption of CIP on GS and on CM, as well as CIP reduction, which can be mediated by the presence of the nanomaterials, justifying the high extent of removal verified in this assay, in comparison with biotic.CIP, where RM are not present. In the abiotic.CNT.CIP, CIP removal after the third cycle is much lower than under the other conditions, about 2.8-fold lower, probably because there is only one mechanism of CIP removal present, adsorption on CNT, which are in a low concentration (0.1 g L^{-1}) and saturates faster than the GS. It is important to note that a dynamic adsorption/desorption process on biomass and on CM may also occur during the incubation period.

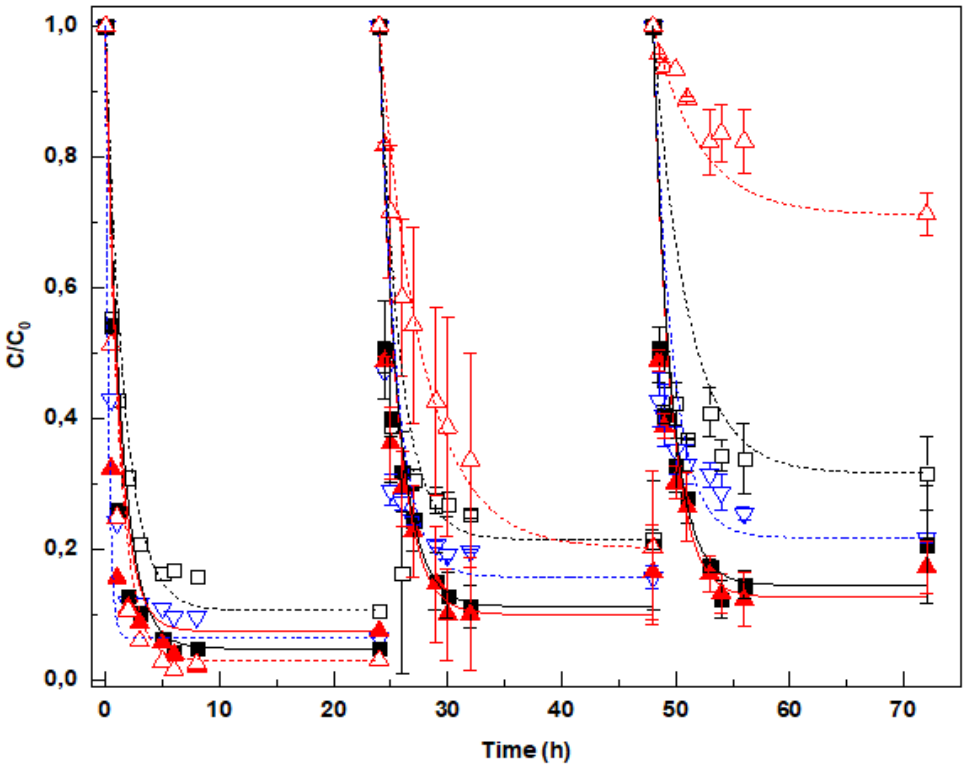

Figure 6. Ciprofloxacin removal over three cycles of 24 h of CIP addition. The results include biologic without CNT and without substrate (Blank.CIP □); biologic with CNT and without substrate (Blank.CNT.CIP ▽); biological without CNT (Biotic.CIP ■); biological with CNT (Biotic.CNT.CIP ▲); abiotic without sludge (Abiotic.CNT.CIP △).

Table 5. Extent (%) and rate (d^{-1}) of removal of the ciprofloxacin at the end of each cycle of 24 hours

Samples	Cycle 1		Cycle 2		Cycle 3	
	Removal (%)	Rate (d^{-1})	Removal (%)	Rate (d^{-1})	Removal (%)	Rate (d^{-1})
Blank.CIP	90	27.8	79 ± 2	22.0 ± 5.1	68 ± 6	25.8 ± 3.2
Blank.CNT.CIP	94	64.8	84 ± 3	25.6 ± 0.7	78 ± 1	23.7 ± 3.6
Biotic.CIP	96 ± 1	40.1 ± 8.9	89 ± 3	33.3 ± 9.3	86 ± 2	33.9 ± 3.5
Biotic.CNT.CIP	98 ± 1	53.7 ± 8.0	90 ± 9	35.8 ± 4.1	88 ± 4	36.1 ± 5.3
Abiotic.CNT.CIP	96 ± 1	20.6 ± 18.9	80 ± 8	7.2 ± 3.1	29 ± 3	3.1 ± 1.2

Ethanol consumption and acetate formation were also followed by HPLC in the different assays. The results show equal patterns of the oxidation of ethanol to acetate and the totally consumption of the substrate at each cycle, in all conditions, except in the abiotic control (annex IV). As expected, in abiotic conditions, the substrate is not consumed, so the accumulation of ethanol is verified. No differences in the substrate consumption, between biotic conditions with and without CNT, proving that in this concentration, CNT does not affect the microorganism's activity [79].

Methane production in biological assays was measured along the assays by GC and the results are presented in annex V. No significant differences were observed for the different conditions, so, similarly to the verified and discussed above for AO10 assay, the presence of CNT does not affect the activity of microorganisms.

4. CONCLUSIONS AND FUTURE PERSPECTIVES

In this work different CM were tested as RM in AO10 decolourisation, namely surface modified CM (CNT_HNO_3 and CNT_MB_M) and impregnated with iron (CNT@2%Fe, CNT@2%Fe_HNO_3 and CNT@2%Fe_MB_M). For this purpose, biologic assays with granular sludge, abiotic and blank controls with and without CM were performed. Additionally, following a previous work, CNT were applied as RM in the removal of CIP, conducting three cycles of CIP addition, in order to saturate the GS and CNT during the first cycle(s), so highlighting the mechanism of CIP removal, once in previous work performing only one high adsorption on CNT and on granular sludge was observed.

AO10 decolourisation increased in the presence of all CM tested, when compared with biological control without CM, where 29±3 % of AO10 removal is reached. It can be concluded that all the CM used act as RM in biologic decolourisation of the dye, providing fast electron transfer and accelerating the reduction reaction. Nevertheless, differences between CM performances in AO10 removal was revealed. The best results were obtained in the biological assay in the presence of 0.1 g L^{-1} of CNT_MB_M: dye removal of 98±1 % at the rate of 2.94±0.18 d^{-1}. These results suggest that the modifications obtained in this CNT, namely the incorporation of N atoms (that can provide additional electrons) and the increase of S_{BET} and V_p (favoring the approximation of the dye to CM), make the reduction of the dye faster.

Like AO10, most of the azo dyes used in the textile industry are anionic. So, in the future, the main goal is preparing CM with additional surface modification, in order to increase their pH_{pzc} to more basic values, making them more favorable materials for adsorption and electron transfer by favoring the electrostatic interaction, probably resulting in a better decolourisation of the AO10 and also of other anionic dyes.

In the removal of CIP, after the third cycle of CIP addition, the differences between the different conditions are visible, showing the saturation of the CM and GS and biological removal. The removal of CIP under abiotic conditions, showed a decrease of 29±3 % of CIP due to adsorption on CNT and of 68±6 % due to the adsorption of GS (under blank condition) in the third cycle. In the preceding cycles the adsorption was higher than 80%, so it is evident that in the last cycle conducted the materials and GS are being saturated.

Despite the saturation of CNT and GS, the removal of CIP in the biological assay remained high (88±4 %) so evidencing the biological removal of the antibiotic and proving the presence of three mechanisms for CIP removal that may occur simultaneously: adsorption of CIP on CNT, adsorption of CIP on GS and biological reduction of CIP.

The effect of CNT as RM was not as evident as for AO10 and only slight increase of the rate was observed.

In future works, carrying out more cycles of CIP removal in order to completely saturate CNT and GS is suggested, so as to evidence better differences between all conditions and the effect of CNT as RM. Also, surface modified CM should be tested as RM for CIP removal, tailoring the CM for this compound, and finding the better RM, given the correlation between properties of the compound and the surface of CM. Testing different modified CM, including magnetic one, for other pharmaceuticals, and model and real wastewaters containing these pollutants, is also proposed, in order to understand if this method of treatment is applicable to the general biodegradation of pharmaceuticals. Pharmaceutical removal in a continuous reactor with the best CM selected by the batch experiments is another future work with the important aiming of achieve a full-scale application.

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ANNEX I – AO10 CALIBRATION CURVE

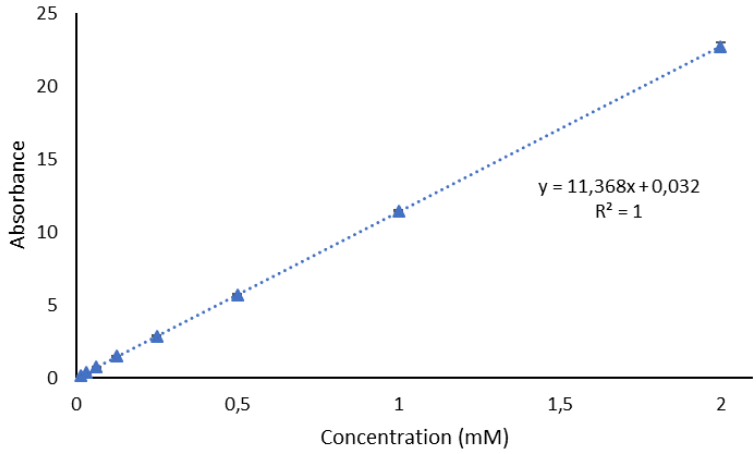

Figure I.1. Calibration curve for AO10, obtained by spectrophotometry, at 480 nm.

ANNEX II – CIP CALIBRATION CURVE

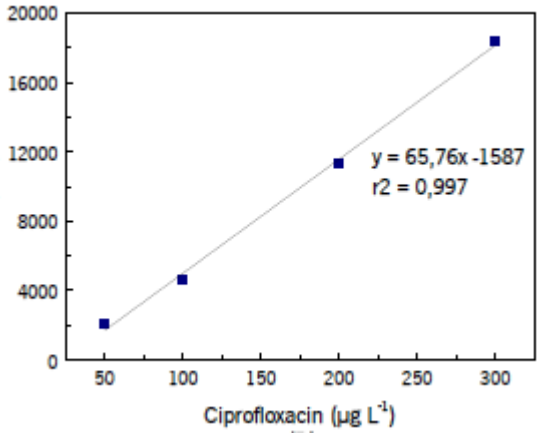

Figure II.1. Calibration curve of CIP, obtained by HPLC.

ANNEX III – CIP REMOVAL

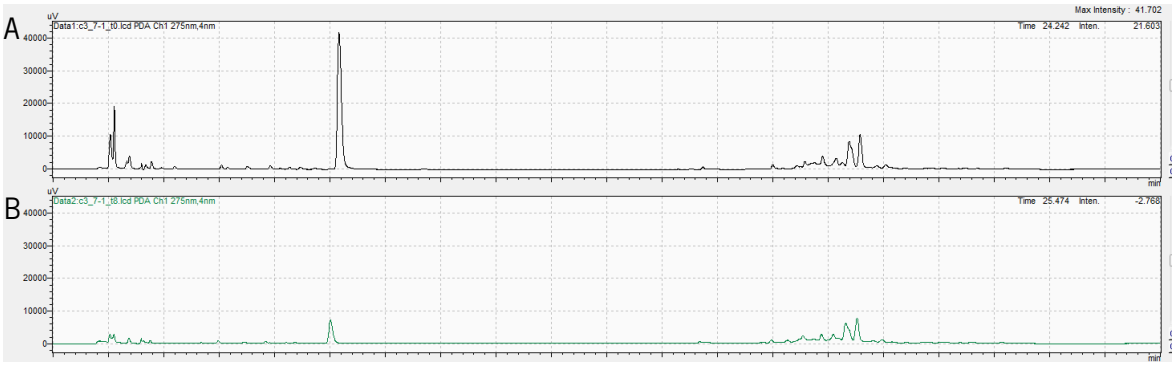

Figure III.1. HPLC chromatograms at 275 nm of CIP removal assay under biotic condition with CNT (biotic.CNT.CIP) at (A) 0h of reaction and (B) 8h of reaction.

ANNEX IV – SUBSTRATE CONSUMPTION AND CONVERSION ALONG 3 CYCLES OF CIP BIODEGRADATION

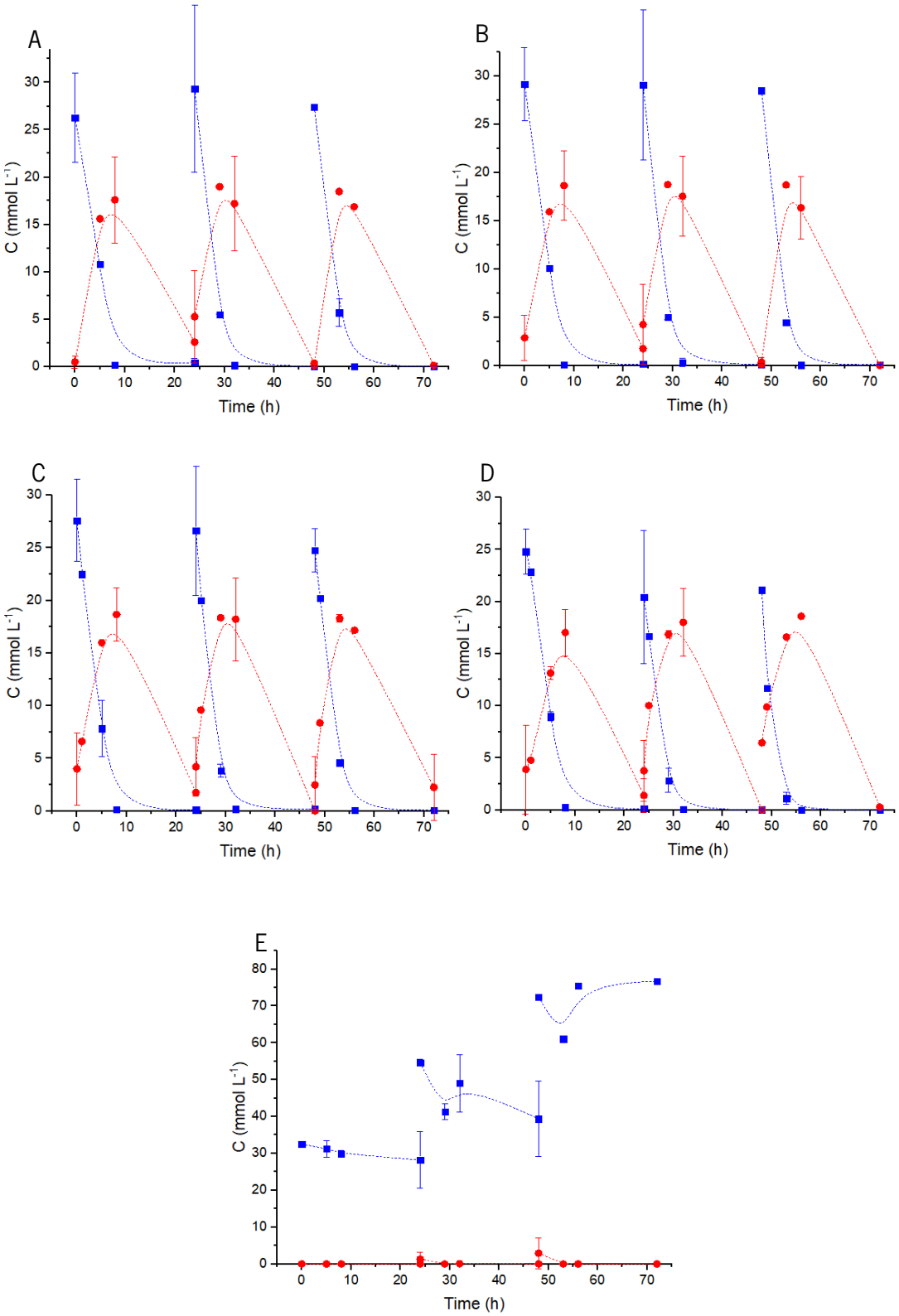

Figure IV.1. Consumption of ethanol (■) and conversion to acetate (●) during the biological process, in the (A) biotic, (B) biotic.CIP, (C) biotic.CNT, (D) biotic.CNT.CIP and (E) abiotic.CNT.CIP conditions.

ANNEX V – METHANE PRODUCTION ALONG 3 CYCLES OF CIP BIODEGRADATION

Table V.1. Concentration of methane (mmol L^{-1}) before 24 hours of each cycle of CIP biodegradation and respective production rate (mmol L^{-1} h^{-1}). In blank assays production of methane is not applicable

Samples	CH_4 (mmol L^{-1})			Production rate$_{CH4}$ (mmol L^{-1} h^{-1})		
	Cycle 1	Cycle 2	Cycle 3	Cycle 1	Cycle 2	Cycle 3
Biotic	43.5 ± 0.4	51.5 ± 0.7	50.9 ± 0.06	2.58 ± 0.05	2.78 ± 0.06	3.03 ± 0.03
Biotic.CIP	44.8 ± 1.3	50.4 ± 0.8	51.3 ± 0.5	2.61 ± 0.03	2.89 ± 0.03	3.00 ± 0.06
Biotic.CNT	44.7 ± 0.7	51.5 ± 0.1	50.3 ± 0.7	2.62 ± 0.04	2.84 ± 0.03	3.07 ± 0.03
Biotic.CNT.CIP	44.4 ± 0.6	50.7 ± 0.6	47.9 ± 0.8	2.51 ± 0.08	2.86 ± 0.03	2.92 ± 0.03