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Innovative Strategies for the Disinfection of Drinking Water Distribution Systems: Antimicrobial Potential of Fenton Reaction

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"Se nos detivermos a pensar nas pequenas coisas

chegaremos a compreender as grandes."

José Saramago

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Abstract

The protection of public health against bacterial contamination of drinking water distribution systems (DWDS) remains one of the main worldwide concerns. Access to drinking water (DW) is a good health requirement and a human right, so it is essential to find innovative and effective strategies to control the microbial growth in DWDS.

The aim of this dissertation was to understand the effects of Fenton reagent against both planktonic and biofilm cells of *Escherichia coli* and to optimize Fenton reaction conditions, in order to create an efficient treatment against bacteria under conditions mimicking DWDS. For growth control of *E. coli* in its planktonic state, cells were exposed to different pH (at 2, 3, 4, 5 and 7), iron dosages (10, 20, 50 and 100 mM) and hydrogen peroxide concentrations (1, 2, 5, 10, 20, 50 and 100 mM) for a maximum of 3 hours of treatment. The mode of action of the Fenton reagent with planktonic bacteria was assessed through the analysis of physicochemical characteristics of the bacterial surface, cellular viability and membrane integrity. For biofilm formation were used two different types of bioreactors: polystyrene microtiter plates (24 h-old biofilms) and a well-stirred continuous reactor with stainless steel and polystyrene coupons (5 d-old biofilms).

In planktonic assays, the Fenton treatment showed to be more effective at acidic pH, with higher doses of catalyst and hydrogen peroxide and for longer exposure times. The optimum Fe^{2+}:H_2O_2 molar ratio was 2:1 (20 mM:10 mM) after 3 hours of treatment at pH = 3 (7-log CFU reduction). With respect to bacterial surface characterization, *E. coli* cells became hydrophobic after exposure to the Fenton reaction and more hydrophilic when the acidic pH was the only variable. Moreover, Fenton reaction caused remarkable cell membrane damage, allowing the uptake of propidium iodide (100% stained cells). Regarding the behaviour of biofilm cells, these were more resistant to disinfection by Fenton reaction than planktonic cells. While the acidic pH caused the major effects in the disinfection of 24 h-old biofilms (2-log CFU reduction), neither the Fenton reaction nor the acidic pH showed influence in the bacterial inactivation of 5 days-old biofilms.

Hereupon, the Fenton reaction was efficient in the disinfection of suspended cells, moderately effective against young biofilms (24 h-old) and not effective against mature biofilms (5 days-old).

Resumo

A proteção da saúde pública contra a contaminação bacteriana dos sistemas de distribuição de água potável continua a ser uma das principais preocupações mundiais. O acesso a água potável é um requisito para a boa saúde e um direito humano, deste modo é essencial descobrir estratégias inovadoras e eficazes para controlar o crescimento microbiano nos sistemas de distribuição.

O objetivo desta dissertação foi compreender os efeitos do reagente de Fenton contra ambas as células planctónicas e de biofilme de *Escherichia coli* e otimizar as condições da reação de Fenton, de modo a criar um tratamento eficiente contra bactérias sob condições que mimetizam os sistemas de distribuição de água potável. Para o controlo do crescimento de *E. coli* no seu estado planctónico, as células foram expostas a diferentes pH (a 2, 3, 4, 5 e 7), dosagens de ferro (10, 20, 50 e 100 mM) e concentrações de peróxido de hidrogénio (1, 2, 5, 10, 20, 50 e 100 mM) para um máximo de 3 horas de tratamento. O modo de ação do reagente de Fenton com células planctónicas foi avaliado através da análise das características físico-químicas da superfície bacteriana, viabilidade celular e integridade da membrana. Para a formação de biofilme, foram utilizados dois diferentes tipos de biorreatores: microplacas de poliestireno (biofilmes de 24 h) e um reator em modo contínuo com agitação e com cupões de aço inoxidável e de poliestireno (biofilmes de 5 dias).

Nos ensaios planctónicos, o tratamento de Fenton mostrou ser mais eficaz a pH ácido, com doses mais elevadas de catalisador e de peróxido de hidrogénio e para maiores tempos de exposição. O rácio molar ótimo de Fe^{2+}:H_2O_2 foi 2:1 (20 mM:10 mM) após 3 horas de tratamento a pH = 3 (redução de 7-log UFC). Em relação à caracterização da superfície bacteriana, as células de *E. coli* tornaram-se hidrofóbicas após exposição à reação de Fenton e mais hidrofílicas quando a única variável foi o pH ácido. Além disso, a reação de Fenton causou danos notáveis na membrana celular, permitindo a incorporação de iodeto de propídeo (100% de células coradas). Relativamente ao comportamento das células do biofilme, estas foram mais resistentes à desinfeção por reação de Fenton do que as células planctónicas. Enquanto que o pH ácido causou os principais efeitos na desinfeção de biofilmes de 24 h (redução de 2-log UFC), nem a reação de Fenton nem o pH ácido mostraram ter influência na inativação bacteriana dos biofilmes de 5 dias.

A reação de Fenton foi eficiente na desinfeção de células em suspensão, moderadamente eficaz contra biofilmes jovens (24 h) e não foi eficaz contra biofilmes maduros (5 dias).

Declaração

Declara, sob compromisso de honra, que este trabalho é original e que todas as contribuições não originais foram devidamente referenciadas com identificação da fonte.

Assinar e datar

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Glossary

AOPs – Advanced oxidation processes

ATR – Acid tolerance response

BOD - Biochemical oxygen demand

CFU – Colony-forming units

COD – Chemical oxygen demand

DW – Drinking water

DWDS – Drinking water distribution systems

EPS – Extracellular polymeric substances

H_2O_2 – Hydrogen peroxide

HClO – Hypochlorous acid

L/D – Live/Dead® *Bac*Light™ bacterial viability kit

·OH – Hydroxyl radical

PCA – Plate count agar

PI – Propidium iodide

SS – Stainless steel

TVS – Total volatile solids

UV – Ultra-Violet

VBNC – Viable but nonculturable

Chapter 1

1. Work Outline

1.1. Background and project presentation

Access to safe drinking water (DW) is a fundamental requirement for good health and is also a human right. However, approximately one-half of the world's population experiences diseases that are the direct consequence of polluted DW. One of the main risks to health comes from the ingestion of water contaminated with faeces containing pathogens that cause infectious diseases such as cholera and enteric fevers. Such illness are the primary cause of infant mortality in many Third World countries (WHO 2011; Bain et al. 2014).

Despite modern progress in science and engineering, the disinfection of water distribution infrastructures, known as drinking water distribution systems (DWDS), remains an important worldwide concern (WHO 2014). Some of the threats to the integrity of DWDS are pipe aging, biocorrosion, biofilm formation and persistence of pathogen microorganisms (Berry et al. 2006). Biofilms result from the growth of bacteria that can thrive in DWDS. In bacteria that are adapted to low-nutrient conditions, as verified in DWDS, traditional treatments with chlorine or chloramines as disinfectants are unable to eradicate the microorganisms from the DW. Besides chlorination, there is a wide range of end-of-pipe technologies available for the treatment and microbiology control of DWDS. However, the majority of these technologies have high energy consumption, high operation costs, can lead to excessive production of sludge and might create new environmental problems (O'Connor 2002; Crittenden et al. 2012). Therefore, there is a pressing need to create innovative and effective strategies to overcome these limitations.

Among the existing advanced oxidative processes (AOPs), Fenton reaction is presented as one of the most promising oxidation techniques for the reduction of toxic organic pollutants and has been currently studied to control bacterial growth in DWDS (Gosselin et al. 2013a; Ascenção 2014).

1.2. Main objectives

The main objectives of this work were to understand the effects of Fenton reagent against *Escherichia coli* and to optimize Fenton reaction conditions to create an efficient treatment against bacteria in both planktonic and biofilm states under conditions mimicking drinking water distribution systems (DWDS).

First, in planktonic assays, the effect of different parameters on *E. coli* growth control were study such as pH (at 2, 3, 4, 5 and 7), iron dosage (10, 20, 50 and 100 mM), hydrogen peroxide concentration (1, 2, 5, 10, 20, 50 and 100 mM) and also the reaction time to better assess reaction kinetics.

To study the aspects of the mode of action of the Fenton reagent with planktonic bacteria, the hydrophobicity of cells was determined through the measurement of contact angles by application of the sessile drop method. Additionally, the study of cellular viability and membrane integrity was made through the Live/Dead *Bac*Light bacterial viability kit.

By using the previous optimized conditions in the planktonic tests, biofilm assays were performed in 96-well polystyrene microtiter plates and in a well-stirred continuous reactor to form 24 h-old and 5 d-old biofilms, respectively. In the reactor, biofilm growth occurred on stainless steel and polystyrene coupons.

1.3. Thesis organization

This work is organized in five chapters. Chapter 1 describes the main goals of this study and the outline of this dissertation.

Chapter 2 provides a brief literature review focusing on the contaminant factors of DWDS, the limitations of the available technologies for end-of-pipe wastewater treatment and the role of Fenton reaction mechanism in the treatment of DWDS. It also includes an analysis of the factors that have major influence on the Fenton process, the contribution of Fenton treatment in microbial growth control and a comparison between different approaches of Fenton-like processes.

Chapter 3 describes the study of the effects of pH value, iron dosage, hydrogen peroxide/iron ratio and Fenton reaction time in planktonic cells of *E. coli*. The study of

bacterial surface characteristics, permeability and cellular viability is also described through the measurement of contact angles and by epifluorescence microscopy inspections with the Live/Dead *Bac*Light stains.

Chapter 4 shows the behaviour of 24 h-old biofilms when exposed to different variations of Fenton reagent and also the effect of Fenton optimized conditions in 5 days-old biofilms formed on stainless steel and polystyrene coupons.

Chapter 5 contains the general conclusions withdrawn from this work and some remarks for future work.

Chapter 2

2. Literature review

2.1. Drinking water concerns

Providing safe drinking water (DW) to the world population has always been an important health and development issue at national, regional and local levels. In some regions, it has been shown that investments made in water supply and sanitation can yield a net economic benefit, provided that the reductions in adverse health effects and health care costs outweigh the costs of undertaking the interventions. Particularly, interventions in improving access to safe DW in underdeveloped countries can also be an effective part of poverty alleviation strategies (WHO 2011).

In 2006, UNICEF and WHO estimated that 1.1 billion people had no access to improved water supplies and 2.6 billion people lacked adequate sanitation (Moe and Rheingnas 2006). In some areas where there is no connection to main sewage systems the alternative is to build septic systems. Even in 2007, an estimated 20% of total U.S. housing units were served by septic systems, also referred to as onsite systems to treat wastewater. A septic tank is a primary treatment system which involves only separation of solids and some preliminary anaerobic action. Usually, tanks are installed below-ground and homeowners are often responsible for maintaining their system. If properly executed, it can protect public health and preserve valuable water resources but many local authorities no longer allow septic tanks to be installed, instead requiring anaerobic or advanced treatment systems which provide a higher level of treatment (EPA 2008).

Not only the septic systems but also the overall distribution systems, which are a critical component of every DW utility, constitute a significant management challenge from both an operational and public health standpoint. Consequently, preventing the aging of water infrastructures is one of the top water priorities in the USA and in many other industrialized countries. EPA research is focusing on increasing the life of DW and wastewater systems, determining the causes of system failures and finding ways to prevent future breakdowns (Gutierrez 2014).

2.2. Drinking water distribution systems and contamination factors

Distribution systems represent the vast majority of physical infrastructure for water supplies, however, there are several problems that can threaten their safety and thereby reducing the water-quality provided. With the aging of these systems, deterioration can occur due to corrosion, materials erosion and external pressures that can lead to breaches in pipes and storage facilities (NAS 2006). Concerning the microbiological aspects, the main problems in the distribution systems networks are biofilm formation, nitrification, biocorrosion and the persistence of some pathogen microorganisms in bulk water (EPA 2002a; Berry et al. 2006; Simões et al. 2010; Wahman and Pressman 2014). DW biofilms represent a complex microbial community (bacteria, fungi, protozoa and also virus and helminths) embedded in a heteroexopolymer matrix mainly made of proteins and polysaccharides that provides cohesiveness to the system (Wingender and Flemming 2011). The process of biofilm formation will be described in more detail in section 4.1 but it is noteworthy that this process depends on different factors such as: type of pipe materials, diversity of microorganisms present in the microbial community, temperature and pH values of the surrounding environment, concentration of residual disinfectants and of nutrients and also the fluid dynamics and hydrometric parameters of the distribution systems networks (Simões 2012).

In extreme situations, for example, during an emergency in which there is evidence of faecal contamination of the DW systems, immediate measures should be made to prevent major damages to the health of consumers. The following sections address the available technologies for end-of-pipe water/wastewater treatment, the Fenton reaction mechanism and its importance in the disinfection of DWDS and a comparison between different approaches.

2.3. Available technologies for end-of-pipe water/wastewater treatment

End-of-pipe treatment is, by definition, not pollution prevention. However, it is an important aspect of pollution control and it sometimes competes financially with pollution prevention options. This type of treatment is called “end-of-pipe” because

usually represents the last stage of a process before the stream is disposed or released to the environment (Guyer 1998). The aim is to modify the residual products of production processes so that they are less damaging to the natural environment than untreated residual products (Zotter 2004). Several reports emphasize different strengths of the end-of-pipe treatments using various strategies such as confining the contaminants in a defined area (e.g. landfilling), reducing toxic effects by dilution (e.g. smoke-stacks), transferring pollutants from one medium to another (e.g. air-stripping of contaminated water) or converting pollutants to inert materials (e.g. mineralization) (Cervantes et al. 2006).

In wastewater treatment, end-of-pipe systems are essential for meeting the defined limits for different parameters such as colour, biochemical oxygen demand (BOD), chemical oxygen demand (COD) and pollutants concentration. Current methods can be divided into four categories: physical, chemical, biological and thermal methods. Physical methods include processes related strictly with physical phenomena and are used, for example, to remove suspended solids from the wastewater. They include sedimentation, screening, aeration, filtration, flotation, degasification and equalization (Curran 2006). They can also be used for removing organic and inorganic matter in colloidal suspension by ultra and microfiltration. Chemical methods rely upon the chemical interactions between the pollutant compounds and the applied chemicals used to improve the water quality. Among the chemical technologies are chlorination, ozonation and neutralization. Chlorination will be further discussed in section 2.6. Regarding the application of biological methods, they resort to the use of microorganisms, mostly bacteria, to generate stable end products that will deposit and will be later removed from the effluent. Generally, biological treatment methods can be divided into aerobic and anaerobic methods based on the availability of dissolved oxygen. Activated sludge, aerobic lagoons and biologic rotary discs are included in aerobic methods as opposed to anaerobic lagoons, anaerobic digestion and septic tanks. Finally, thermal methods can be applied as a means of sterilization, for example, to sterilize sludges contaminated with organic contaminants (EPA 2000; Cheremisinoff 2002; Tünay 2010). Additionally, for metal removal, the non-standard methods used include ion exchange, evaporation and microfiltration (Cushnie 2009).

Although end-of-pipe control technologies have contributed to reduce negative environmental problems of industrial processes, they also have several disadvantages: do not focus in the origin of the environmental problem, are not adequate to allow an efficient use of limited resources, can cause high consumption of materials and energy and consequently have higher operation and maintenance costs and their use generally creates new environmental problems (Cervantes et al. 2006).

In order to overcome the inflexibility of some of the above-mentioned end-of-pipe technologies and also the complexity of combined wastewaters, in particular due to the presence of refractory organic compounds (e.g. pesticides, phenols, surfactants), there is a need for the use of advanced oxidation processes (AOPs) to effectively treat the effluents. Among them, Fenton system (Fe^{n+}/H_2O_2) is one of the most promising oxidative techniques for the reduction of refractory and toxic organic pollutants in water and wastewater treatment (Badawy and Ali 2006; Badawy et al. 2009; Ribeiro et al. 2015).

2.4. Fenton reaction mechanism

The Fenton reaction was first developed by Henry Fenton for the oxidation of tartaric acid in the presence of iron (Fenton 1864). Most recently, the Fenton treatment was regarded as an inexpensive and environmentally friendly technology widely used for treating wastewater (Guimarães et al. 2014). It is also used for remediation of contaminated solid matrices such as soils, sediments or sludges (Luong and Lin 2000; Vicente et al. 2011).

The process generates powerful oxidative species such as hydroxyl radicals (· OH, with a redox potential of 2.73 mV under acidic conditions) through a series of reactions involving iron and hydrogen peroxide, at acidic pH values. The mechanism of Fenton's oxidation involves mainly the following steps (equations 1-8):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH \quad (1)$$

$$\cdot OH + H_2O_2 \rightarrow H_2O + HO_2^{\cdot} \quad (2)$$

$$H_2O_2 + Fe^{3+} \rightarrow Fe^{2+} + H^+ + HO_2^{\cdot} \quad (3)$$

$$Fe^{3+} + HO_2^{\cdot} \rightarrow Fe^{2+} + H^+ + O_2 \quad (4)$$

$$Fe^{2+} + HO_2^{\cdot} \rightarrow Fe^{3+} + HO^- \quad (5)$$

$$\cdot OH + Fe^{2+} \rightarrow OH^{-} + Fe^{3+} \quad (6)$$

$$RH + \cdot OH \rightarrow R\cdot + H_2O \quad (7)$$

$$R\cdot + Fe^{3+} \rightarrow R^{+} + Fe^{2+} \quad (8)$$

In this process, iron (Fe^{2+}/Fe^{3+}) acts as catalyst and RH concerns to the organic pollutants. Due to the complexity of the process, besides the above-mentioned reaction steps many others are described in literature, however, for some of them the rate constants vary from work to work (Herney-Ramirez et al. 2009).

The hydroxyl radicals produced can react with pollutants according to four different ways: (i) addition of ·OH to the structure of the contaminant; (ii) hydrogen absorption; (iii) electron transmission and (iv) interaction between free radicals leading to the formation of stable compounds (Mansoorian 2014). The main effects of the high reactivity of these hydroxyl radicals are organic matter degradation, decrease of toxicity, increase of biodegradability, decrease of BOD, COD and total organic carbon (TOC), and removal of colour and odour (Selvakumar et al. 2009).

Research on the use of the Fenton's reagent has increased in the past few years and its efficiency for water/wastewater treatment has been studied under several pH and reaction conditions. The process is known to be very sensitive to pH, ratio of Fe^{2+} to H_2O_2 and the concentration of the catalyst (Fe^{2+}) and oxidant (H_2O_2). Additionally, the temperature of the environment, the type of substrate used and amount of organic matter present have also a considerable influence in the process (Kang and Hwang 2000). Several studies showed that the kinetics of global reaction with Fenton's reagent is a first order reaction with respect to the concentration of organic substrate in the wastewater (Lucas and Peres 2006; Fares Al et al. 2008; Medien and Khalil 2010).

The use of Fenton (H_2O_2/Fe^{2+}) and modified Fenton or Fenton-like (H_2O_2/Fe^{3+}) methods have greater advantages among the range of the known advanced oxidation processes, such as simple operation, low reaction time, non-toxic compound production, economic viability, and possibility of utilization at different scales (Mansoorian 2014). Besides, it runs at moderate conditions of temperature and pressure.

Regarding some of the different parameters that have major influence in the oxidation process, the first one to take into account is the pH. In general, optimum pH values for the homogeneous Fenton process occur between 3 and 5 (Gosselin et al.

2013a) but there are several studies contradictory on this matter and a more precise and narrow range of optimum values is difficult to find. The decrease in reaction efficiency for pH values higher than 5 is due to the transition of hydrated Fe^{2+} ions to colloidal ferric species, $Fe(OH)_3$. The iron precipitate as $Fe(OH)_3$ and will decompose catalytically the hydrogen peroxide to oxygen and water, thereby decreasing the formation of hydroxyl radicals (Selvakumar et al. 2009). Below the optimum pH (~3), the hydroxyl radicals are scavenged by protons and the degradation efficiency declines. In order to maintain this value, large amounts of acid (usually sulphuric acid) must be added to the reaction medium (Valdés-Solís et al. 2007).

Another essential parameter is the temperature of the surrounding environment. It has been shown that the oxidation reaction rate increases substantially with the increase of the temperature. Typically, optimum temperature values for operation are between 20 and 50 ºC (Haddad 2014). Wu et al. (2010) reported that the oxidation removal efficiency of humic acid increased linearly from 61% to 81% when the temperature increased up to 45 ºC. In contrast, by increasing the temperature from 45 to 55 ºC the yield of the process was reduced. This phenomenon is explained by two opposite effects of the temperature on the reaction yield. First, higher temperature values enhanced the generation rate of ·OH and therefore enhanced the oxidation efficiency. However, extremely high temperature, over 55 ºC, led to the acceleration of hydrogen peroxide decomposition into oxygen and water thus decreasing the oxidation efficiency of the humic acid (Wu et al. 2010). Besides of economic criteria for using so high temperatures, safety factors should also be taken into consideration.

In the absence of the iron catalyst, normally as a solution of $FeSO_4$ or by adding iron minerals or other iron/metal sources to the samples, the formation of hydroxyl radicals does not happen. Matta et al. (2007) tested different iron minerals (ferrihydrite, hematite, goethite, lepidocrocite, magnetite and pyrite) for the degradation of TNT (2,4,6-trinotrotoluene) and reported that pyrite and magnetite were more effective than the ferric oxides. The better efficiency of pyrite catalyst over magnetite was due to the high ability of pyrite to dissolve into water in the presence of hydrogen peroxide, oxygen and Fe^{3+} species. Even so, the effect of ferric and ferrous dosage on the chemical removal efficiency depends from case to case. Fenton efficiency can be enhanced by increasing the Fe^{2+} and Fe^{3+} concentration which accelerate the ·OH

generation rate and consequently enhance the oxidation rate of the pollutants. After reaching a certain concentration, the removal efficiency is not enhanced (Mansoorian 2014) and in some cases an abrupt decrease of degradation was observed since high Fe^{2+} concentrations favoured the occurrence of scavenging reactions (equations 4, 5 and 6 above-mentioned) (Wu et al. 2010).

The use of chelating agents can enhance degradation efficiency of iron minerals at near neutral pH because of their positive effects on the dissolution rate of iron oxide (Pouran et al. 2014). Xue et al. (2009) investigated the effect of EDTA, carboxymethyl β-cyclodextrin (CMCD), oxalate, tartrate, citrate and succinate as chelating agents with magnetite on pentachlorophenol (PCP) decomposition rate. All applied chelating agents promoted iron-oxide dissolution amount and PCP oxidation rate.

The dosage of H_2O_2 also plays an essential role in the Fenton process. It is commonly used as a biocide, particularly in applications where its decomposition into non-toxic by-products is important. As suggested in general reviews on the mechanisms of action of biocides, H_2O_2 is considered an oxidizing agent reactive with biomolecules (e.g. proteins, lipids, nucleic acids) that make up cellular and viral structure/function (Linley et al. 2012). Nevertheless, free ·OH radicals have a much higher oxidation potential compared to H_2O_2 alone (Bigda 1995). When the concentration of H_2O_2 increases, the time required for the complete reaction gets shorter. But similarly to the iron catalyst, when H_2O_2 is present in excess in the system it acts as a scavenger of the ·OH radicals to produce perhydroxyl radicals (·OOH) – equation 2 above-mentioned – which have much lower oxidation capabilities than ·OH radicals (Wu et al. 2010).

Zhang et al. (2005) showed that the efficiency of the Fenton process was improved by adding Fenton's reagent in multiple steps rather than in a single step. Additionally, they also stated that the reaction kinetics was highly dependent on the H_2O_2/ Fe^{2+} molar ratio. Typical Fenton catalyst/H_2O_2 molar ratios range between 1:5 to 1:25 (Lewinsky 2007).

Generally, the four main steps involved in industrial homogeneous Fenton's oxidation processes are (i) oxidation reaction, (ii) neutralization and coagulation (iii) flocculation and (iv) sedimentation (Bigda 1995; Mansoorian 2014). At the end of the oxidation stage is required to neutralize the reaction acidic mixture. Due to the existence

of iron in the solution, the pH raise leads to the formation of iron hydroxides that precipitate originating sludges (Gosselin et al. 2013a).

2.5. Fenton in microbial growth control

The conventional approach to biological control in distribution systems, normally maintaining a disinfectant residual, is often ineffective at controlling microbial growth. The study of the ecology of microbial interactions can help to understand the persistence of pathogens in DW (Berry et al. 2006).

Bacteria exist in two principal forms, as free-floating planktonic cells and in biofilms. Planktonic organisms are important components of many aquatic ecosystems and exhibit a wide range in form and function. They are the link to higher trophic levels. However, it is now known that the majority of microbial cells live in distinct communities, named as biofilms (Paraje 2011). It is also known that disinfection with chlorine can reduce the concentration of planktonic bacteria (cells suspended in the water phase) but have almost no effect on the concentration of biofilm bacteria (Behnke and Camper 2012).

Oxidative stress of bacteria is caused by an imbalance between the production of oxidants, such as free radicals and peroxide, with the levels of antioxidant defences (Paraje 2011). For biofilm disinfection, the aim of Fenton treatment is to generate free radicals in the biofilm or in the surrounding media to unbalance the metabolic pathways of the microorganisms. The high reactivity of ·OH with the organic matter can alter the nutrient availability and the oxygen and osmolarity levels of the surrounding environment damaging cellular components, including the biofilm matrix, DNA, proteins and lipids, and leading to rapid inactivation of bacteria (Paraje 2011; Gosselin et al. 2013a).

As mentioned in the previous section, the oxidative damage of cells by H_2O_2 finds many applications. For example, H_2O_2 has been used to reduce macrofouling in marine cooling water systems, particularly as Fenton's reagent when combined with ferrous ions (Kim 2015). Miller et al. (1996) also observed that the direct application of the Fenton reagent to microorganisms resulted in their death. Other experiments made by

Gosselin et al. (2013b) showed a decrease of almost 55% of attached cells in 9-month-old DW biofilms after treatment with heterogeneous Fenton reagent.

Although the majority of the experiments in this field were made in well controlled conditions, the use of Fenton's reagent and its variants has been shown to be a promising approach to inactivate bacteria as both suspended or surface attached cells.

2.6. Comparison between approaches

The generation of ·OH species can be also obtained by using different variations of the chemical Fenton process. The **electro-Fenton** (E-Fenton) approach has been widely studied for the destruction of organic and biorefractory pollutants contained in wastewaters by highly oxidative hydroxyl radicals formed from the reaction of electrogenerated H_2O_2 with Fe^{2+} (Zhou et al. 2013). It offers some advantages as compared to the traditional chemical Fenton process maintaining the high efficiency of Fenton's reagents (e.g. H_2O_2) and saving costs from the chemical use (oxidant is generated *in situ*), storage and transportation. However, more energy is consumed due to the use of electricity as the main energy source (Ganzenko et al. 2014). The bioelectro-Fenton (Bio-E-Fenton) concept is thus possible by using bioelectrons produced from the microbial metabolism to drive an E-Fenton process. This can be achieved by properly configuring a micro fuel cell reactor which consists of two chambers separated by a cation-exchange membrane: an anaerobic anode chamber filled with biodegradable organic substrates and an aerated cathode chamber with biorefractory pollutants. The electrons are released from the bioreactions at the anode and transported to the cathode through an external load circuit. The reduction of oxygen at the cathode results in H_2O_2 formation, which consequently reacts with the source of Fe^{2+} to produce hydroxyl radicals that will degrade the pollutants (Feng et al. 2010).

Another approach to the generation of hydroxyl radicals is by using radiation through a process named **photo-Fenton**. It can be powered by sunlight with wavelengths of $\lambda < 580$ nm and the same concentration of radicals is obtained by using a lower iron concentration (compared to the chemical process). In addition to the above-mentioned equations, the formation of hydroxyl radicals in photo-Fenton occurs by the following equations (eq. 9 and 10) (Lucas and Peres 2006; Herney-Ramirez et al. 2010):

$$H_2O_2 + UV \rightarrow \cdot OH + \cdot OH \quad (9)$$

$$Fe^{3+} + H_2O + UV \rightarrow \cdot OH + Fe^{2+} + H^+ \quad (10)$$

Even so, the conventional Fenton treatment is more advantageous in terms of costs of operation and maintenance than treatments involving H_2O_2/UV, Fe^{3+}/H_2O_2/UV and TiO_2/UV (Klamerth et al. 2013).

There are many reports describing the use of **supported iron** in a porous matrix made of advanced synthetic and natural materials such as carbon nanotubes, graphene, activated carbon, clays, zeolite and nanocomposites (Hsueh et al. 2006; Martínez et al. 2007; Pham et al. 2009; Herney-Ramirez et al. 2011; Duarte et al. 2012). This is another alternative as opposed to the use of iron catalyst in solution. The heterogeneous solid catalysts can mediate Fenton-like reactions over a wide range of pH values and avoid iron loss because the iron species are "immobilized" within the matrix and the catalyst can maintain its ability to generate hydroxyl radicals preventing the precipitation of iron hydroxide (Garrido-Ramírez et al. 2010). Gan and Li (2013) stated that the synthesis of these advanced materials could be quite tedious and hazardous chemicals and harsh reaction conditions are required. Instead of the above mentioned materials, they suggested the use of a silica supported iron-based catalyst as a more stable, environmentally friendly and low cost material to make the Fenton treatment affordable. However, several other materials can be employed, as stated above.

It has also been suggested that the performance of supported Fe catalysts is often improved by adding certain metals. **Copper ion** has an important role as an assistant in photocatalysis. Guimarães et al. (2009) reported that doping the copper ions into goethite catalyst led to strong increase in its catalytic activity. It has been presented that the outstanding catalytic activity of bimetal catalysts may be generally attributed to synergistic effects in relation with creation of defects, novel active sites and ease redox interplay between copper and iron redox complexes (Han et al. 2011)

The traditional strategy to disinfect drinking water is **chlorination**. Chlorine is the most popular disinfectant used for DW and it is added either as chlorine gas or as sodium hypochlorite (liquid bleach) (Postigo and Richardson 2014). The hypochlorous acid (HClO) is the compound that actually does the disinfection and it is formed when chlorine dissolves in water. Comparing to the redox potential of ·OH (2.73 mV under

acidic conditions), the redox potential of HClO is lower and equal to 1.49 mV. Regarding other disadvantages of this method, chlorination may lead to the formation of disinfection by-products by the reaction of free chlorine with humic substances in water creating a major issue on the balancing of the toxicodynamics of the chemical species and increasing the risk of pathogenic microbes in the supply of DW (Gopal et al. 2007). Moreover, there are evidences indicating that some microbial communities gained resistance to chlorination (Berry et al. 2006).

2.7. Challenges

The main challenge of the Fenton treatment is the unfeasibility to apply homogeneous Fenton processes to *in situ* environmental remediation since large amounts of ferric hydroxide sludges would be produced (result of the pH adjustment of the acidic medium) and consequently generating other environmental problems (Garrido-Ramírez et al. 2010).

Some other challenges are presented in the topics below (Gosselin et al. 2013a):

- A significant decrease in the pH value can endanger the integrity of the pipes of the distribution systems networks (pH < 3).
- For low contents of iron and copper in biofilms, Fenton reactions have limited effects.
- The half-time of ·OH is short in water (<70 ns) as well as the diffusion length (<10 nm), thus for inactivation they should be generated close to microorganisms.
- Possible interference of free radical scavengers.
- Deactivation of iron ions due to their bonding with various reagents such as anion phosphate.
- Release of corrosion products.
- Some organic compounds are not oxidized (e.g. acetic acid and acetone).
- Formation of other compounds which are toxic and that will compromise the applicability of the treatment.
- The removal of sludge leads to an increase in the treatment costs.

Chapter 3

3. Homogeneous Fenton reaction applied to *Escherichia coli* planktonic cells

3.1. Introduction

Bacterial safety of DW cannot be determined by testing samples for all individual pathogens. Since it is impractical and expensive, usually the presence of faecal pathogens in DW is assessed by monitoring for indicator bacteria. The World Health Organization (WHO) recognizes *Escherichia coli* as the indicator of choice, although thermotolerant coliforms can also be used as an alternative (WHO 2011).

Most strains of *E. coli* are harmless and live in the intestines of healthy humans and animals, however, some of them produce powerful toxins and can cause severe illness. One of the best studied and well-known is *E. coli* O157:H7 (Croxen et al. 2013). As well as in other faecal coliforms, *E. coli* propagation occurs during rainfalls and other types of precipitation which can lead to DW contamination if the water is inadequately treated (EPA 2002a; Frenzen et al. 2005; Bruhn and Wolfson 2008). Hereupon, Fenton reaction processes can be important in controlling bacterial proliferation in DWDS.

Concerning the constituents of the Fenton reagent, several studies stated that the major portion of the toxicity of H_2O_2 against *E. coli* is attributed to DNA damage mediated by ·OH radicals. These radicals are in turn originated through the reaction above-mentioned with the iron catalyst (equation 1 in section 2.4). Damage by Fenton oxidants may occur at the DNA bases or sugars. Sugar damage is initiated by hydrogen abstraction from one of the deoxyribose carbons and the main consequence is eventual strand rupture and base release. To minimize the toxicity of oxygen radicals, the cell uses scavengers of these radicals and DNA repair enzymes (Imlay et al. 1988; Henle and Linn 1997; Imlay 2003; Imlay 2008).

Lipids are also major targets during oxidative stress. Free radicals can react directly with polyunsatured fatty acids in membranes and initiate lipid peroxidation.

The process initiates with the abstraction of a hydrogen atom from a phospholipid carbon-carbon double bond creating a carbon radical and consequently a chain reaction that results in abstraction of hydrogen atoms from other lipids until the propagations reaction damages an integral membrane protein. Another effect of lipid peroxidation is a decrease in membrane fluidity, which alters membrane properties and might damage membrane binding sites significantly (Cabiscol et al. 2000; Repetto et al. 2012).

All amino acid residues of proteins are also susceptible to oxidation by ·OH. The multiple sites that can be damaged are often categorised into backbone or amino acid side-chain sites. In the polypeptide backbone, the oxidative attack is initiated via hydrogen atom abstraction at the α-carbon site leading to the formation of a carbon-centred radical. These species can react with other radicals and through a series of subsequent decompositions the result is backbone fragmentation (Berlett and Stadtman 1997; Davies 2005). For both cases, the main protein modifications observed are: loss of catalytic activity, amino acid modifications, carbonyl group formation, increase in acidity, decrease in thermal stability, change in viscosity, change in fluorescence, fragmentation, formation of protein-protein cross-links, formation of S-S bridges and increased susceptibility to proteolysis (Cabiscol et al. 2000; Stadtman and Levine 2003).

Although several efforts have been employed in order to understand protein damage (Hohn et al. 2013; Chondrogianni et al. 2014), there is a dominance of lipid and DNA oxidation studies over proteins. This can be explained by the complexity of proteins since there are 20 different types of amino acids side-chains plus the backbone as potential targets for reactive oxygen species (Nold and Wennemers 2004; Davies 2005).

The aim of this study was to evaluate the effects of different dosages of Fenton reagent components – iron and hydrogen peroxide – and to assess the influence of pH and Fenton reaction time in planktonic cells of *E. coli*. Bacterial surface characteristics and cellular integrity and viability were also considered through the analysis of contact angles and Live/Dead *Bac*Light kit, respectively.

Regarding the cellular viability study presented later in this chapter, it is taken into account that unlike normal cells that are culturable on suitable medium and develop as colonies, viable but nonculturable (VBNC) cells are living cells that have lost the ability to grow on solid medium, on which they normally grow. If VBNC cells are

present, the total number of viable bacteria in a sample will be underestimated by the colony-forming units count method due to the inherent non-culturability of VBNC cells. For bacterial species causing human infections, the underestimation or non-detection of viable cells in water distribution systems may pose serious risks to the public health (Li et al. 2014).

3.2. Materials and methods

3.2.1. Quantification of biomass reduction depending on pH value, iron dosage, hydrogen peroxide concentration and reaction time

To study the efficiency of Fenton treatment against faecal pollution, *E. coli* CECT434 was selected as model microorganism and used to test different pH values (at 2, 3, 4, 5 and 7), iron dosages (10, 20, 50 and 100 mM) and hydrogen peroxide concentrations (1, 2, 5, 10, 20, 50 and 100 mM).

Initially for pH effect assessment, *E. coli* was grown overnight in Luria-Bertani broth medium (Liofilchem, Italy) using an incubator at 30 ºC and 150 rpm (KS 130, IKA®, Germany). After saline (0.85% NaCl) wash and centrifugation (15 min and 3220 g), the absorbance was measured at 600 nm (V-1200 Spectrophotometer, VWR, USA) and set to 0.5 for a final volume of 15 mL using the appropriate buffer. Buffer solutions were prepared with pH values at 2 (using 0.2 M hydrochloric acid/potassium chloride buffer), 3, 4 and 5 (using respectively 0.12, 0.14 and 0.15 M of citric acid/sodium phosphate buffer) and 7 (using 0.04 M of phosphate buffer). For each condition three flasks were used: (i) cell control with 0.85% saline solution, (ii) pH control with only buffer solution and (iii) Fenton reaction containing the buffer with Fenton reagent species (hydrogen peroxide and iron salt). In the third flask were added 20 mM of dissolved iron sulphate ($FeSO_4.7H_20$, Merck, Germany) and 10 mM hydrogen peroxide (H_2O_2, Merck). Every 15 minutes, 1 mL of sodium sulphite (Na_2SO_3, Merck, Germany) – final concentration of 0.13 g/L required to stop the Fenton reaction (Gosselin et al. 2013a) – was added to the three solutions during a reaction time of 1 hour. After the addition of Na_2SO_3 to test tubes and the respective dilutions (10^0-10^{-5}) to have 20 to 200 CFU (colony-forming units) per plate (Figure A1, Appendix), 10 µL of each tube was added in duplicate to PCA (Plate Count Agar, Oxoid, UK) plates. The plates were

maintained at room temperature for 24 h before CFU counts. After pH assessment, the same procedure was used to study the effects of the variation of iron and hydrogen peroxide dosages.

Three other controls were tested using three different flasks (OD_{600} = 0.5), each containing 15 mL of 0.85% saline solution. For iron control were added 100 mM of $FeSO_4.7H_2O$ and for hydrogen peroxide control and sodium sulphite control were added 10 mM of H_2O_2 and 0.13 g/L of Na_2SO_3, respectively.

3.2.2. Bacterial surface characterization through the measurement of contact angles

Cell growth was assessed in the same conditions as described in 3.2.1. After saline wash (15 min and 3220 g), the absorbance was measured at 600 nm (V-1200 Spectrophotometer, VWR, USA) and set to 0.5 for a final volume of 60 mL. Through a filtration system with a cellulose ester membrane with a pore size of 0.45 µm and 47 mm of diameter (Whatman), were filtered 60 mL of *E. coli* suspension for each condition tested (cell control, pH control and Fenton reaction). After filtration, each membrane with the *E. coli* layer was cut cautiously in 6 portions and attached to glass slides with double-sided tape to create a flat surface to favour the measurement of contact angles.

A goniometer (Theta Lite, Biolin Scientific, Sweden) was used to measure the contact angles by the sessile drop method. The samples were carefully placed in the base and each drop was made manually with a micropipet programmed to release 4 µL each time. The determination of contact angles was made immediately after drop collocation through an image analysis system (One Attension, Biolin Scientific) connected to a portable computer. The measurements were carried out at room temperature (23 ± 3 ºC) using three different liquids, water, α-bromonaphtalene and formamide (Sigma, Portugal). Liquids surface tension parameters were obtained from literature (Janczuk et al. 1993).

The degree of hydrophobicity of a specific material (*i*) is expressed as the free energy of interaction between two entities of that material when immersed in water (w) – ΔG_{iwi} (mJ/m^2). This property can be positive or negative. In the case of $\Delta G_{iwi}^{TOT} > 0$,

the material is considered hydrophilic because the interaction between the two entities is weaker than the interaction of each entity with water. As opposed, if $\Delta G_{iwi}^{TOT} < 0$, the material is considered hydrophobic. Hydrophobicity was evaluated using the approach of van Oss et al. (Van Oss et al. 1988; 1989) and calculated through the surface tension components of interacting entities according to equation 11:

$$\Delta G_{iwi}^{TOT} = -2\left(\sqrt{\gamma_i^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_i^+\gamma_w^-} + \sqrt{\gamma_i^-\gamma_w^+} - \sqrt{\gamma_i^+\gamma_i^-} - \sqrt{\gamma_w^+\gamma_w^-}\right) \quad (11)$$

where,

γ^{LW} is the Lifshitz-van der Waals component of the surface free energy;

γ^+ and γ^- correspond to the electron acceptor and donor, respectively, of the Lewis acid-base component (γ^{AB}), with $\gamma^{AB} = \sqrt{\gamma^+\gamma^-}$.

The surface tension components of the surfaces were obtained by the measurement of contact angles of the previous mentioned three pure liquids and by resolution of equation 12.

$$(1 + \cos\theta)\gamma_i^{TOT} = 2\left(\sqrt{\gamma_s^{LW}\gamma_i^{LW}} + \sqrt{\gamma_s^+\gamma_i^-} + \sqrt{\gamma_s^-\gamma_i^+}\right) \quad (12)$$

where, θ is the contact angle and $\gamma^{TOT} = \gamma^{LW} + \gamma^{AB}$.

3.2.3. Determination of viable cells and cell integrity

Three conditions were tested: (i) cell control – cells in 0.85% saline solution; (ii) pH control – cells in 0.12 M citric acid buffer solution with a pH value at 3 and (iii) Fenton reaction – cells in the same buffer solution with 10 mM H_2O_2 and 20 mM $FeSO_4.7H_2O$. After Fenton treatment, cells were stained using a Live/Dead® *Bac*Light bacterial viability kit (Molecular Probes) according to the manufacturer's recommendations. Samples were observed in a fluorescence microscope Leica DM LB2 (Leica Microsystems, Germany). Viable (intact membrane) and nonviable (damaged membrane) cells were differentiated by their signatures in a plot of green (SYTO 9™) fluorescence versus red (propidium iodide) fluorescence, respectively. These stains differ in their ability to penetrate the membrane of bacterial cells. When used alone, SYTO 9™ stain labels both live and dead bacteria. In contrast, propidium iodide (PI)

penetrates only bacteria with damaged membrane, reducing SYTO 9™ fluorescence when both dyes are present.

The percentage number of nonviable cells, marked as red by PI, was obtained according to equation 13:

$$\%\ PI\ stained\ cells\ = \frac{concentration\ of\ red\ cells}{Initial\ concentration} \times 100\% \quad (13)$$

The number of viable but nonculturable (VBNC) cells was determined through the difference between the number of cells obtained by the viability kit and the number of CFU.

3.2.4. Statistical Analysis

Data was analyzed using One Way ANOVA from the statistical software SPSS 20.0 (Statistical Package for the Social Sciences) assuming a significance level for the separation set at $p < 0.05$ and the calculations were based on a confidence level of 95%.

3.3. Results and Discussion

3.3.1. Influence of pH and Fenton reaction time

There are many reports that have successfully modelled the kinetics and species behaviour in simple Fenton systems (Kwan and Voelker 2002; Duesterberg et al. 2008). However, there continues to be some debate regarding the exact nature of pH effects on iron catalysis and overall oxidation performance (Jung et al. 2009). In order to find the most favourable conditions of the Fenton reaction for microbial growth control in DWDS, the first purpose of this work was to analyze the effects of pH on the performance of Fenton-mediated oxidation by exposing *E. coli* cells to a range of solutions with different pH values (experimental setup in Figure A2, Appendix).

Figure 1 shows the evolution of cells (in a logarithmic scale) in a phosphate buffer solution at pH = 7 for over 1 hour of treatment. Results showed that there is no significant difference between the number of cells in Fenton reaction solution, prepared with 20 mM of $FeSO_4.7H_2O$ and 10 mM of H_2O_2, with the number of cells in both pH

and cell controls (p>0.05). This result is probably due to the fact that the optimum pH for *E. coli* growth is in the range of 6.4 to 7.2 (Holt et al. 1994).

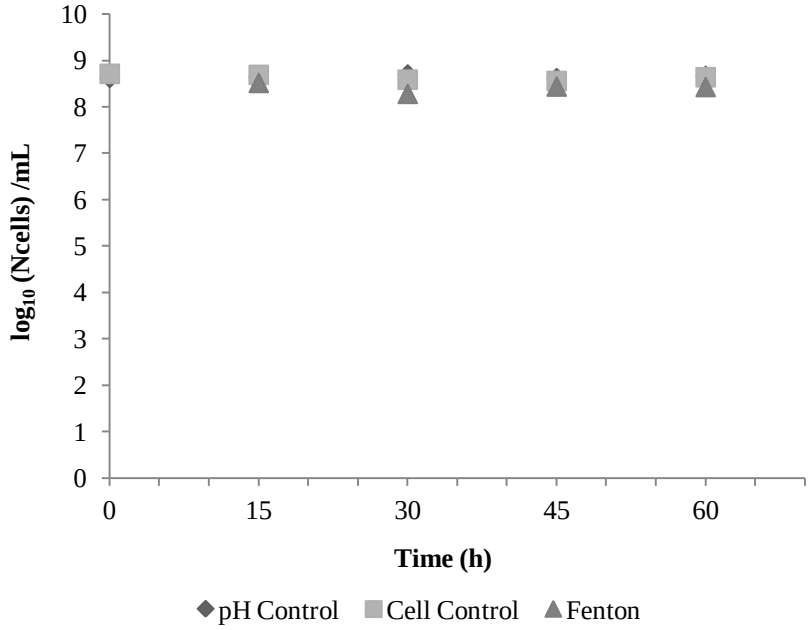

Figure 1. Effects of phosphate buffer at pH = 7 ± 0.2 in the number of cells (logarithmic scale) for different conditions (cell control, pH control and Fenton reaction solution with 20 mM of $FeSO_4.7H_2O$ and 10 mM of H_2O_2) during 1 hour of reaction time. Mean values ± SD for at least three replicates are illustrated.

Another possibility for the absence of variation between the conditions is the precipitation of iron as $Fe(OH)_3$ at neutral pH, that can catalytically decompose the H_2O_2 to oxygen and water, thus blocking the formation of ·OH species (Selvakumar et al. 2009). Moreover, hydrogen peroxide stability is also low at neutral/basic conditions.

Concerning the effects of acidic conditions, when citric buffer with a pH value at 5 was tested (Figure 2), all three conditions showed again almost no variation (p>0.05).

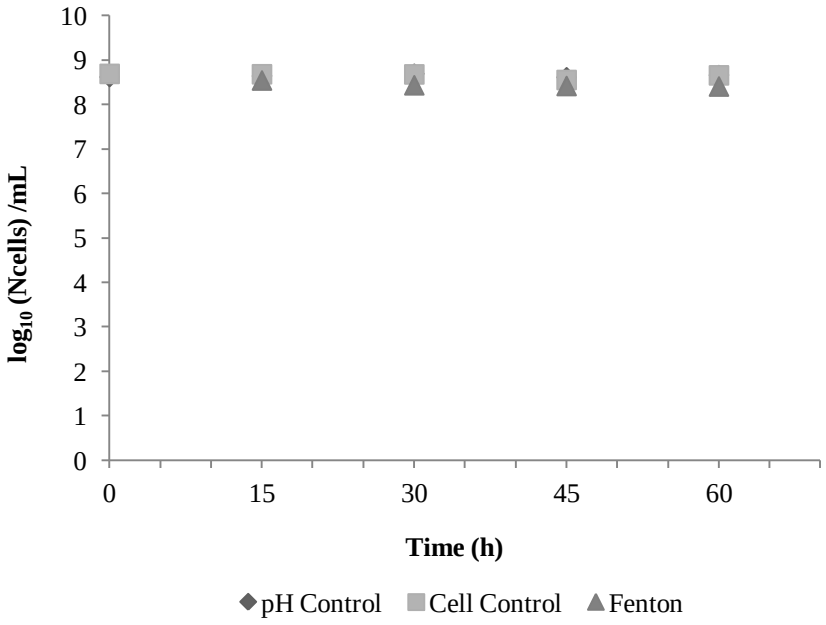

Figure 2. Effects of critic buffer at pH = 5 ± 0.2 in the number of cells (logarithmic scale) for different conditions (cell control, pH control and Fenton reaction with 20 mM of $FeSO_4.7H_2O$ and 10 mM of H_2O_2) during 1 hour of reaction time. Mean values ± SD for at least three replicates are illustrated.

For a better understanding of the reaction kinetics over time, the treatment was thereby extended to 3 hours and samples were analyzed every 30 minutes. Cells were once again exposed to citric buffer at pH = 5 and the results are shown in Figure 3. It was found that even prolonging the treatment there is no visible cellular death. This can be explained by the acid-resistance system existent in *E. coli* which contributes to its survival (Castanie-Cornet et al. 1999; Foster 2004).

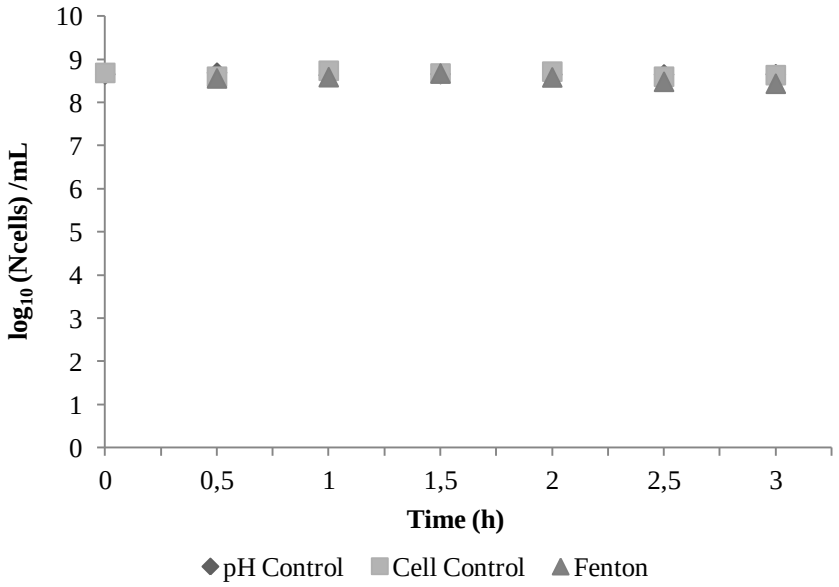

Figure 3. Effect of pH = 5 ± 0.2 (using citric buffer), for 3 hours of reaction, in the number of cells (logarithmic scale) for different conditions (cell control, pH control and Fenton reaction with 20 mM of $FeSO_4.7H_2O$ and 10 mM of H_2O_2). Mean values ± SD for at least three replicates are illustrated.

Tekin et al. (2006) stated that the initial pH value for Fenton treatment has to be in the acidic range of 2 to 4 to generate the maximum amount of ·OH radicals. Furthermore, Cho et al. (2004) showed that the rate of inactivation of *E. coli* is directly proportional to the concentration of ·OH in the system. Following on the influence of acidic pH, buffer solutions with pH values at 2, 3 and 4 were then evaluated.

For pH = 4 (Figure 4) it is evident a gradual decrease in the number of cells exposed to the action of Fenton reagent. Both controls, cells in saline solution and cells in buffer only, showed a constant behaviour over time (p>0.05) proving that the tested buffer alone does not cause cell death. Regarding Fenton reaction, cells decreased significantly from t = 1.5 h (p<0.05) reaching a variation of approximately four orders of magnitude (4-log) at the end of the treatment (t = 3 h).

By decreasing even more the pH value, as shown in Figure 5, the action of Fenton reagent had a more pronounced effect in the bacteria. Similar to pH = 4, there is a significant reduction in the number of cells from t = 1.5 h (p<0.05) but after 3 hours of exposure, the number of surviving cells was only 70 cells per mL of solution (a reduction in a logarithmic scale of about seven orders of magnitude relatively to the controls).

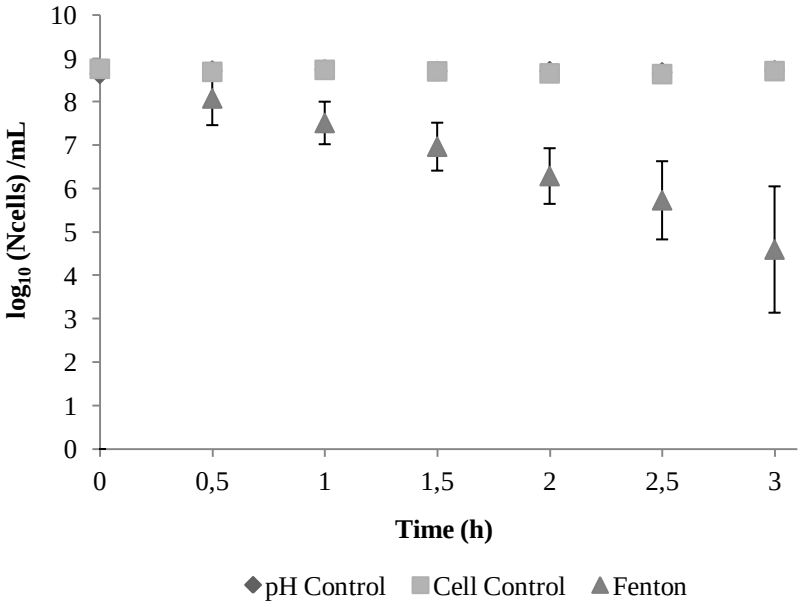

Figure 4. Influence of citric buffer with a pH value at 4 ± 0.2 in the number of cells (logarithmic scale) for different conditions tested (cell control, pH control and Fenton reaction with 20 mM of $FeSO_4.7H_2O$ and 10 mM of H_2O_2) during 3 hours of time reaction. Mean values ± SD for at least three replicates are illustrated.

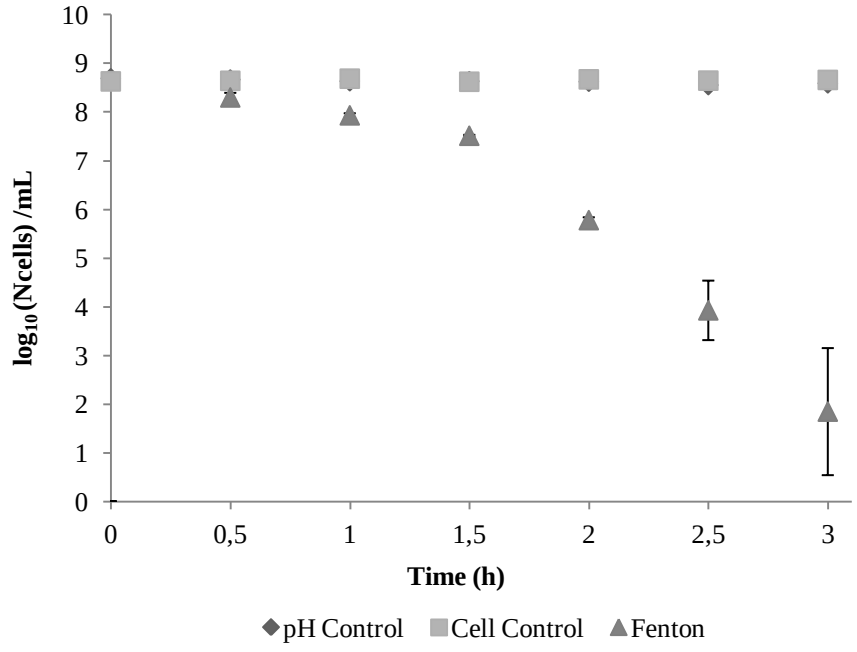

Figure 5. Influence of citric buffer with a pH value at 3 ± 0.2 in the number of cells (logarithmic scale) for different conditions tested (cell control, pH control and Fenton reaction with 20 mM of $FeSO_4.7H_2O$ and 10 mM of H_2O_2) during 3 hours of time reaction. Mean values ± SD for at least three replicates are illustrated.

Kinetic equations for both pH values of 3 and 4 are presented in Figures 6 and 7, respectively. For each condition, the determination of the death rate constant (k_d) was based in a pseudo first-order reaction kinetics.

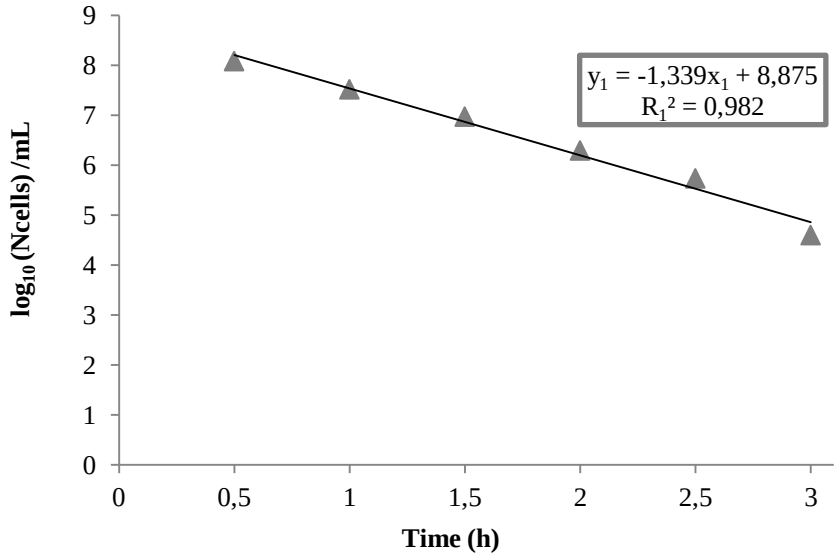

Figure 6. Kinetic equation for the Fenton reaction with 20 mM of $FeSO_4.7H_2O$ and 10 mM of H_2O_2, using citric buffer at pH = 4 ± 0.2. (k_d = 1.339 ± 0.562 h^{-1}, R_1^2 = 0.982). Mean values ± SD for at least three replicates are illustrated.

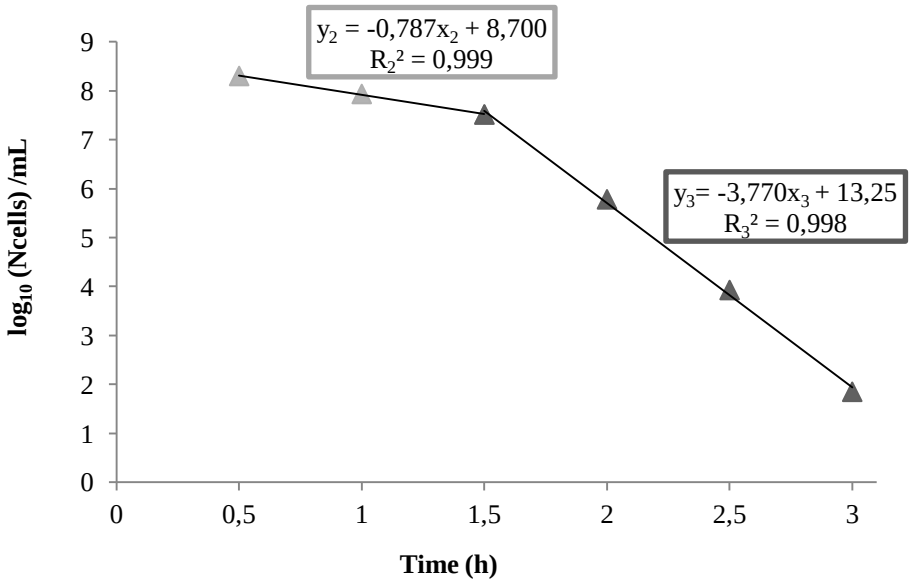

Figure 7. Kinetic equations for the applied Fenton's reaction with 20 mM of $FeSO_4.7H_2O$ and 10 mM of H_2O_2, using citric buffer at pH = 3 ± 0.2. (k_{d2} = 0.787 ± 0.151 h^{-1}, R_2^2 = 0.999; k_{d3} = 3.770 ± 1.764 h^{-1}, R_3^2 = 0.998). Mean values ± SD for at least three replicates are illustrated.

For pH = 4 (Figure 6) the k_d was 1.339 h^{-1} while for pH = 3 the linear fitting was divided into two equations (Figure 7). The first one, from the beginning of the Fenton's

treatment until t = 1.5 h, with an absolute value of k_d of 0.787 h^{-1} and the second, from that point until the end of the reaction (t = 3 h), with an absolute value of k_d of 3.770 h^{-1}. The overall rate of cell death was higher for cells treated with Fenton reagent under lower pH solutions. This can be explained by the fact that the highest concentrations of ·OH are formed at pH = 3, which consequently leads to a higher oxidative stress in the cellular environment followed by fast bacterial inactivation. Moreover, the use of two different kinetic fittings at pH of 3 can be related with the Fenton's process mechanism, as seen in previous works (Herney-Ramirez et al. 2011; Silva et al. 2012).

For pH = 2, there is no graphical representation for the assays conducted under these conditions since no colonies were counted in pH control and Fenton reaction plates. These results suggest that the buffer solution/low pH was the main factor for the total cell death.

Table 1 shows the death rate constants for the acidic pH values tested, the correlation factor for the respective linear fittings and the range values of the number of cells where each equation was applied.

Table 1. Death rate constants (k_d), correlation factors and range values of the number of cells (logarithmic scale) for Fenton reactions performed with pH values at 2, 3 and 4.

pH	k_d (h^{-1})	Correlation factor	Range values (log_{10})
*2**	-	-	-
3	0.787 ± 0.151	R_1^2 = 0,999	7.5 – 8.3
	3.770 ± 1.764	R_2^2 = 0,998	1.8 – 7.5
4	1.339 ± 0.562	R_3^2 = 0,982	4.5 – 8.1
5	0	-	-

*No colonies were found (total cell inactivation).

Additionally, other control solutions were tested in order to assess if, individually, the constituents of Fenton reagent and sodium sulphite, used to stop Fenton reaction, have effect in *E. coli* without the influence of pH. For that, *E. coli* cells were exposed to three saline solutions containing respectively 100 mM of $FeSO_4.7H_2O$, 10 mM of H_2O_2 and 0.13 g/L of Na_2SO_3, and the results are shown in Figure 8. It was confirmed that there was no significant difference in the amount of surviving cells (p>0.05) between the tested solutions and the cell control.

It is well-known that iron plays an essential role in a large number of cellular processes such as storage, transport, activation of molecular oxygen and ATP generation which consequently contributes to cell growth and proliferation (Cabiscol et

al. 2000). In *E. coli*, iron is also needed for DNA synthesis. (Messenger and Barclay 1983). Even so, when high levels of free iron are present, it may damage or destroy the natural resistance of bacteria (Symeonidis and Marangos 2012), which can explain the slight decrease obtained in the number of cells for the iron control (from t = 2 h).

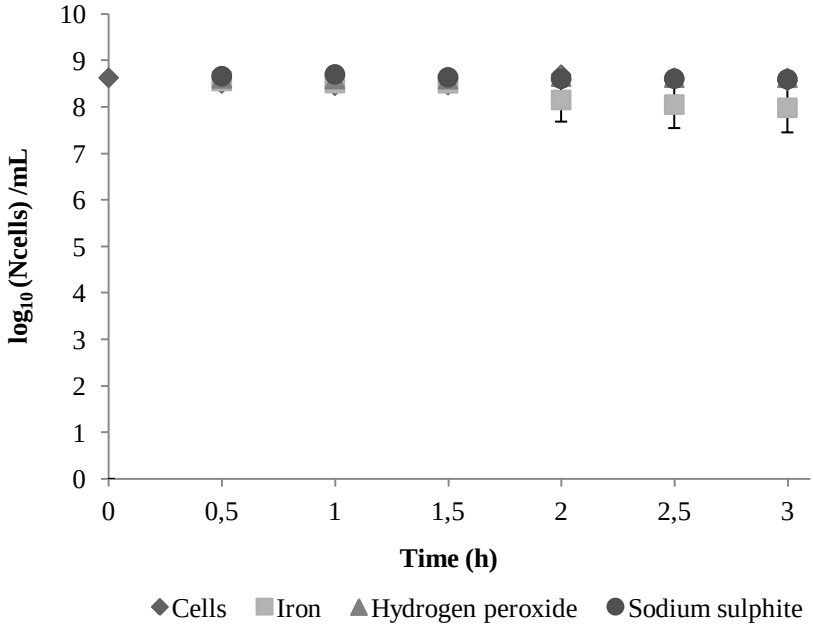

Figure 8. Control conditions tested in saline solution for 3 hours (cell control, iron control with 100 mM of $FeSO_4.7H_2O$, hydrogen peroxide control with 10 mM of H_2O_2 and sodium sulphite control with 0.13 g/L of Na_2SO_3). Mean values ± SD for at least three replicates are illustrated.

3.3.2. Effects of iron and hydrogen peroxide dosages

After pH assessment, the following step in the optimization of Fenton reaction conditions was to study the effect of different dosages of iron (Figure 9) and H_2O_2 (Figure 10) in the inactivation of bacterial cells and to find the most effective molar ratio of Fe^{2+}:H_2O_2.

First, different iron dosages were tested (10, 20, 50 and 100 mM) by setting the concentration of H_2O_2 to 10 mM and by using a citric buffer solution with pH = 3 (Figure 9). In the first 30 minutes of treatment, none of the solutions caused significant variation in the number of cells comparing with pH control (p>0.05). However, 1-2 h after both solutions with 50 and 100 mM of dissolved iron showed a significant reduction in the cell number (p<0.05) until complete bacterial inactivation achieved at t = 2.5 h (noted with symbol "×"). Solutions with 10 and 20 mM started their significant effect from t = 1.5 h (p<0.05) and the latest resulted in higher degrees of cell death. The

reason behind this behaviour is related with the role of iron salt in the Fenton process – Fe^{2+} acts as catalyst, and so the higher is the salt dose, the fastest is the generation of hydroxyl radicals and inherently the bacterial inactivation.

Even though Fenton reactions with 50 and 100 mM of dissolved iron were more effective in inactivating *E. coli* cells than reactions with lower concentrations, the optimal concentration of iron chosen was 20 mM. This is due to the fact that by using sub-lethally stressed cells, the existence of bacterial response is ensured and therefore a more interesting state is created to study in a biological level (Simões et al. 2007).

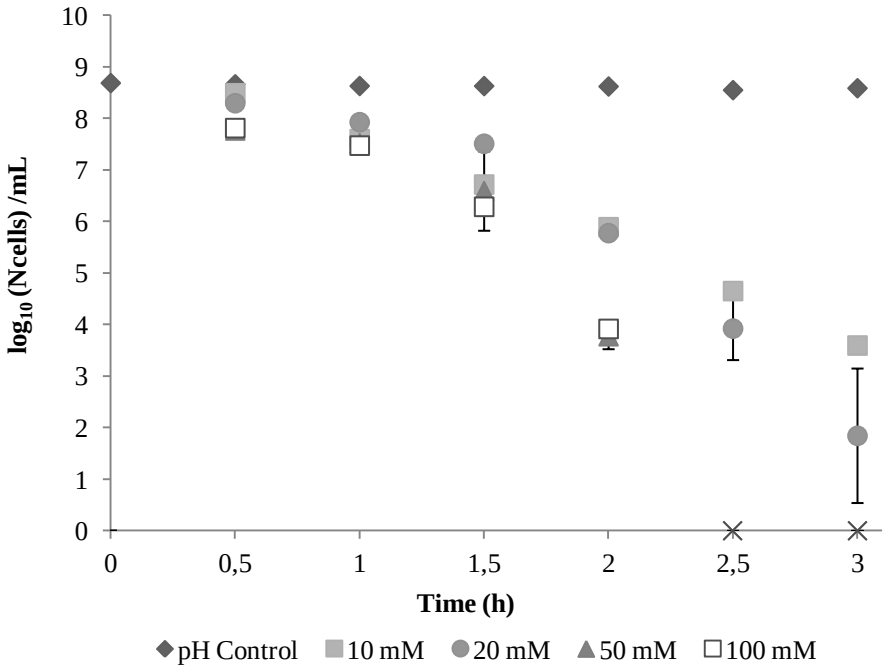

Figure 9. Effects of different iron dosages (10, 20, 50 and 100 mM of $FeSO_4.7H_2O$) in Fenton reaction processes with 10 mM of H_2O_2 and using citric buffer at pH = 3. Mean values ± SD for at least three replicates are illustrated. (×) For values below the method detection limit.

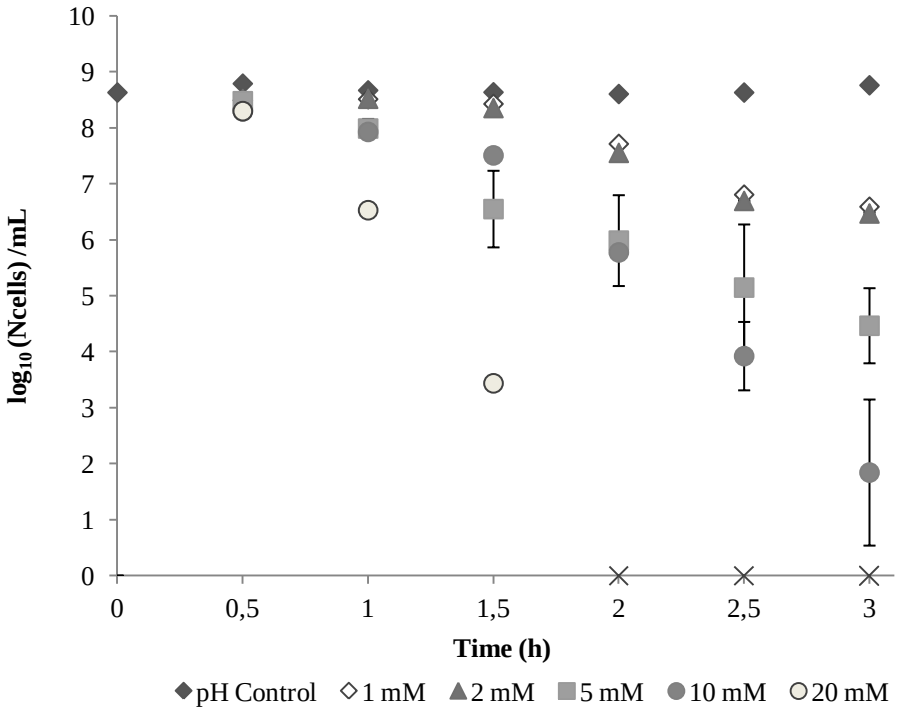

Figure 10. Influence of different hydrogen peroxide concentrations (1, 2, 5, 10 and 20 mM) in Fenton reaction processes with citric buffer solution (pH value at 3) and 20 mM of $FeSO_4.7H_2O$. Mean values ± SD for at least three replicates are illustrated. (×) For values below the method detection limit.

Regarding the optimization of H_2O_2 dose, five concentrations were tested (1, 2, 5, 10 and 20 mM) by fixing the previous optimized iron concentration of 20 mM and by performing the treatment at pH = 3.

As shown in Figure 10, as the concentration increases bacterial inactivation became faster. The highest concentration used, 20 mM, had a significant effect in cell death from 1 hour of treatment (p<0.05) and resulted in total cell death at t = 2 h (noted with symbol "×"). In contrast, lower concentrations, 1 and 2 mM, achieved a reduction of only two orders of magnitude in the number of cells near the end of the treatment. Similarly to iron dosage assays and for the same reason described above, on the subject of sub-lethal conditions (Simões et al. 2007), the selected value for the optimal concentration of H_2O_2 applied to the Fenton reaction was 10 mM.

Combining both studies, the optimum Fe^{2+}:H_2O_2 molar ratio obtained was 2:1 (20 mM:10 mM) which resulted approximately in a 7-log decrease in the number of CFU after 3 hours of treatment at pH = 3.

3.3.3. Bacterial surface physicochemical characterization

For understanding the action mechanisms of the optimized Fenton reaction in *E. coli* cells, surface hydrophobicity of the bacteria was studied by using a methodology proposed by van Oss (1997). This approach allows the determination of the absolute degree of hydrophobicity of material surfaces through surface tension parameters, which can be, in turn, estimated by the measurement of contact angles.

Table 2 shows values of physicochemical parameters (surface tension parameters and hydrophobicity) of *E. coli* surface for the different conditions tested (cells in saline solution, cells in citric buffer at pH = 3 and cells exposed to Fenton reaction with 20 mM of dissolved iron and 10 mM of H_2O_2, performed at pH = 3).

Table 2. Surface tension parameters (γ^{LW} – Lifshitz-van der Waals component; γ_i^+ - electron acceptor; γ_i^- - electron donor) and hydrophobicity (ΔG_{iwi}^{TOT}) of *E. coli* for each condition tested (cell control, pH control and Fenton reaction).

Condition	Surface tension parameters (mJ/cm^2)			Hydrophobicity (mJ/m^2)
	γ^{LW}	γ_i^+	γ_i^-	ΔG_{iwi}^{TOT}
Cell Control	34.4	0	71.6	66.1
pH Control	33.1	0	79.7	74.7
Fenton Reaction	43.7	0	29.1	-0.5

Analyzing Table 2 and according to the classification described by the applied method, *E. coli* was characterized as hydrophilic surface ($\Delta G_{iwi}^{TOT} > 0$ mJ/m^2). In literature, *E. coli* is also regarded for being hydrophilic (Chaves 2004). By exposing the cells to citric buffer (pH control condition) the bacteria surface became more hydrophilic ($p<0.05$) with an increase of the total interaction energy from 66.1 to 74.1 mJ/m^2. This can be due to the higher concentration of H^+ present in the buffer solution at pH = 3, which may have enhanced the hydrophilic character of the bacterial surface. When cells were exposed to Fenton reaction in the citric buffer solution, the total interaction energy drastically decreased ($p<0.05$) and the bacterial surface became hydrophobic ($\Delta G_{iwi}^{TOT} < 0$ mJ/m^2).

Moreover, by comparing the Fenton reaction condition with both controls, the apolar parameter (γ^{LW}) showed that the molecules of the surface became more apolar (from 34.4 and 33.1 to 43.7 mJ/cm^2). The Fenton reaction also decreases the surface electron donor (γ_i^-) character of *E. coli*, while no effect was found on the electron acceptor parameter (γ_i^+). Hereupon, both pH and Fenton reagent significantly interacted with the bacterial surface components resulting in the modification of their physicochemical properties.

3.3.4. Cellular viability

As previously mentioned, the underestimation or non-detection of viable cells of pathogens in water distribution systems can endanger public health (Li et al. 2014). The CFU count method only gives an estimate of the number of cells present; commonly it is an underestimated number since the only cells able to form colonies are those that can grow under the conditions of the method (e.g. incubation media, temperature, time) (Sutton 2011).

In order to overcome the limitations of the plate count method, Live/Dead tests were performed where cells were differentiated by their signatures in a plot of green (SYTO 9™) fluorescence versus red (propidium iodide) fluorescence, respectively.

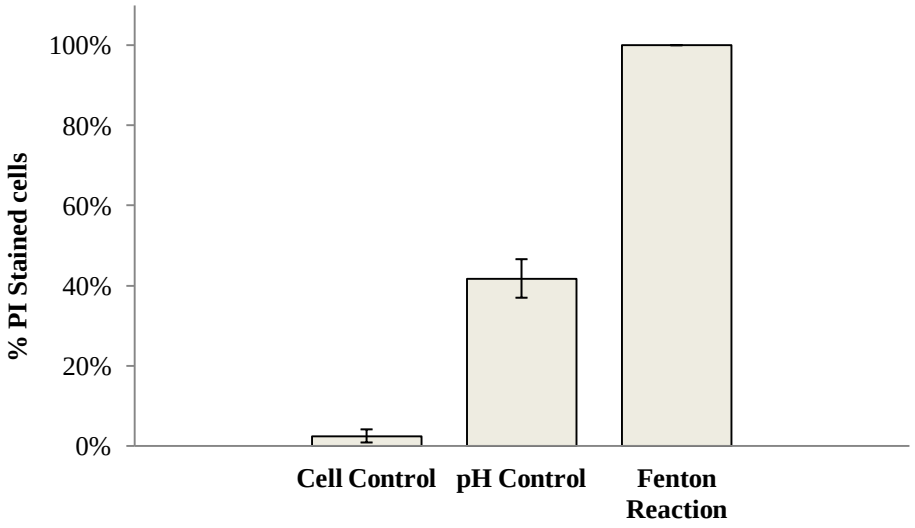

Figure 11. Percentage of nonviable cells (stained with propidium iodide) obtained after 3 hours of treatment for both control solutions – cell and pH – and for a Fenton reaction solution in optimized conditions.

The percentage of nonviable cells, stained with propidium iodide (PI) and marked as red, for all three conditions tested is shown in Figure 11. For cells in saline solution, the percentage of PI stained cells was relatively low (2.4 ± 1.2 %). The existence of nonviable cells were not significant ($p>0.05$) and can be explained by the possible use of intense vortex during the procedure which may have compromised the cell membrane. For pH control, the percentage of PI stained cells increased ($p<0.05$) to 42 ± 5 %. This result proposes that the tested pH caused membrane permeabilization of a significant number of bacteria. However, the Fenton reaction caused the membrane permeabilization of all the bacteria (100% PI stained cells).

While dead cells are metabolically inactive and do not express genes, VBNC cells are metabolically active carrying out respiration and continue transcription thus producing mRNA (Lleò et al. 2000; Besnard et al. 2002). Cells can enter the VBNC state as a response to stressful conditions such as nutrient starvation, use of pH and salinity ranges outside the permissive values to cell growth and extreme temperatures (Cook and Bolster 2007; Cunningham et al. 2009). Nevertheless, when the proper inducer is used VBNC cells may become culturable again (Pinto et al. 2011). It is important to note that treatments normally assumed to be bactericidal, as the chlorination, may instead result in the induction of the VBNC state (Oliver et al. 2005).

In this study, the number of VBNC cells was obtained by subtracting the number of viable cells (marked as green) by the number of CFU. For cell and pH controls, the numbers were respectively $1.55 \times 10^8 \pm 1.06 \times 10^7$ and $2.70 \times 10^7 \pm 1.41 \times 10^6$ VBNC cells per mL. Regarding the Fenton treatment, all cells were marked as red. This result proposes that L/D staining results overestimated the efficiency of the Fenton reaction. In fact, even if all the bacteria had a compromised membrane (100% PI stained cells) 70 CFU/mL were counted.

Regarding the effects of acidic media, known as acidic shock, *E. coli* has been shown to possess an acid tolerance response (ATR) which depends on the stationary growth conditions (Castanie-Cornet et al. 1999). So, when bacterial cells are subjected to extreme acid stress, the main defence strategies are: (i) changes in membrane composition (Yuk and Marshall 2004), (ii) internal pH homeostasis systems (Richard and Foster 2004) and (iii) pathways induced by acid-shock proteins to repair or protect essential cellular components (Seputiene et al. 2006). Even so, for organisms that prefer

growth at neutral pH, such as *E. coli*, the internal pH maintenance of more than two units than the external pH (Δ pH of 2) is nearly impossible. So, when the external pH is 3, the maximum internal pH in cells will be 5, which may inhibit some physiological processes and will begin to denature proteins, DNA depurination and membrane damage (Foster 2004). Antolinos et al. (2014) showed that the cellular viability of *Bacillus cereus* and *B. weihenstephanensis* also decreased at lower pH values and longer exposure times, being the pH at 3.4 the most effective.

In Figure 12 is presented one image obtained by epifluorescence microscopy for the Live/Dead test applied to cells in pH control.

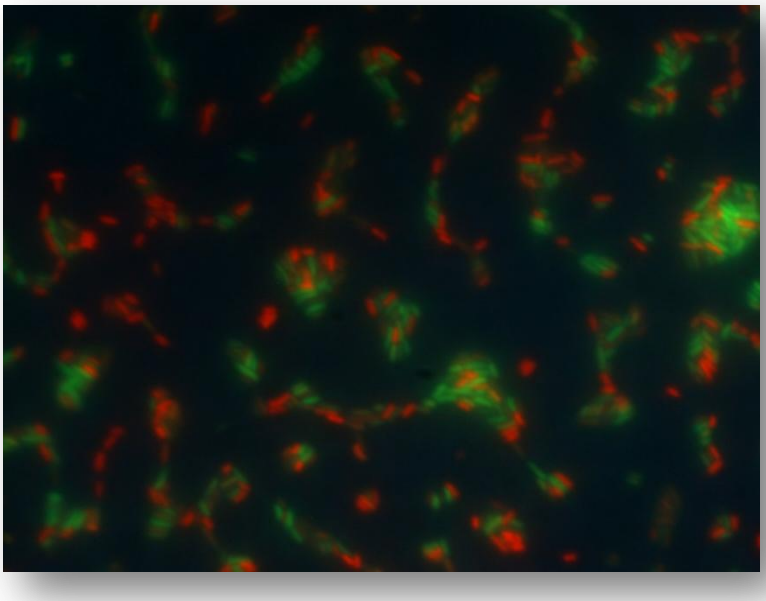

Figure 12. Epifluorescence microscope inspection of *E. coli* stained with the Live/Dead *Bac*Light viability kit after exposure to pH control at 3. Red cells stained with propidium iodide and green cells marked with SYTO 9™.

3.4. Conclusions

For the microbial growth control of *E. coli* in its planktonic state, the Fenton treatment showed to be more effective at acidic conditions, when higher doses of catalyst and hydrogen peroxide are used and with the extension of the reaction time.

By combining the previous studies, the optimum Fe^{2+}:H_2O_2 molar ratio obtained was 2:1 (20 mM:10 mM) which resulted approximately in a 7-log decrease in the number of CFU after 3 hours of treatment at pH = 3.

The exposure of *E. coli* cells to the optimized Fenton reaction changes some characteristics in the bacterial surface components. Planktonic cells of *E.coli*, regarded as hydrophilic, become hydrophobic after Fenton treatment. The electron donor properties of *E. coli* membranes were significantly reduced. Moreover, low pH and Fenton reaction caused cell membrane damage, allowing the uptake of propidium iodide.

Chapter 4

4. Fenton reaction mechanism applied to *Escherichia coli* biofilms

4.1. Introduction

Despite the range of preventive measures available to control water quality, some microorganisms can persist after treatment and gain access to distribution systems. As a consequence, DWDS have been reported to be aquatic ecosystems where a variety of microbial lifestyles can be found, from viruses to protozoa (Szewzyk et al. 2000; Douterelo et al. 2014).

When microorganisms are within DWDS they face a challenging environment with nutrient limited conditions and fluctuations in pressure and water flow. As a result, microorganisms have a better chance of survival if they are attached to the pipe surfaces within a biofilm (Henne et al. 2012). More than 95% of the microbial biomass existing in DWDS is attached to the pipe walls in the form of biofilms (Flemming et al. 2002). They are predominant over planktonic cells being up to 100-1000 times more resistant to disinfectants such as chlorine and other biocides. Biofilm antimicrobial resistance can be explained by multiples mechanisms including an increased number of persistent cells, quorum sensing systems, biosorption, gene expression response and efflux systems (Schembri et al. 2003; Choi et al. 2010; Kurniawan and Yamamoto 2013). Also, protection owing to the production of extracellular polymeric substance (EPS) and limited mass diffusive transport contributes to the antimicrobial resistance (Berry et al. 2006; Stewart and Franklin 2008).

A model for the process of biofilm development is represented in Figure 13. First, at stage 1, bacterial cells attach reversibly to the surface. At the stage 2 occurs the production of EPS resulting in irreversibly attached cells after losing their flagella-driven motility. At stage 3, the first maturation phase is reached. The second maturation phase is reached at stage 4 with fully matured biofilms, as indicated by the complex biofilm architecture. Finally, at stage 5, single motile cells disperse from the biofilm,

reaching a balanced equilibrium between sloughing off and growth events (Stoodley et al. 2002).

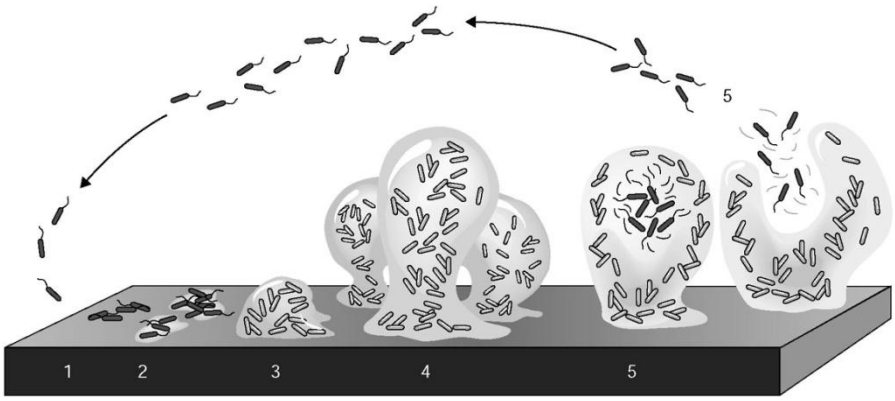

Figure 13. Diagram model showing the five-stages of bacterial biofilm development. Adapted from (Stoodley et al. 2002)

Even if biofilms provide a survival advantage to harmful microorganisms, causing economical costs and increasing the risk of pathogen transmission to consumers, there is no reliable biofilm control strategy causing complete removal and inactivation. In this chapter the Fenton reagent was applied as a new approach to control *E. coli* biofilms, using the conditions optimized in the planktonic tests (chapter 3).

4.2. Materials and methods

4.2.1. Formation of 24 h-old biofilms in polystyrene microtiter plates

To evaluate the efficiency of the previous optimized Fenton treatment conditions in 24 h-old biofilms, *E. coli* CECT434 was first inoculated overnight in Luria-Bertani broth medium using an incubator at 30 ºC and 150 rpm (KS 130, IKA®, Germany). The absorbance was determined at 600 nm (V-1200 Spectrophotometer, VWR, USA) and the bacterial suspension was diluted to achieve an absorbance of 0.04. The suspension was used to fill wells of a 96-well polystyrene microtiter plate (Orange Scientific) with 200 µL and the plate was incubated for 24 hours under agitation at 30 ºC and 150 rpm. Afterwards, the medium was discarded and the wells were washed with 0.85% saline solution. The Fenton reagent was added at the desired concentration (20 µL of Fenton reagent + 180 µL of acid citric buffer solution with a pH value at 3). For the cell control 200 µL of saline solution was added while 200 µL of 0.12 M citric buffer was used for

the pH control. The microtiter plate was then incubated for 3 hours under the same conditions above-mentioned. After the exposure time, the medium was discarded and the wells were washed with 200 µL of saline water. To scrap the biofilm each well was filled with saline solution (250 µL) and the resuspended biofilm was reserved in a 1.5 mL tube. This step was repeated 2 times and 400 µL of saline solution was added to the tube. In all tubes 100 µL of 0.13 g/L of sodium sulphite was also added to stop the Fenton reaction. The dilutions of the biofilm suspension were made in test tubes (10^{-1} to 10^{-5}) in order to obtain 20 to 200 CFU per plate, and each tube was vigorously vortexed before each dilution. After the dilutions, 10 µL of each tube was added in duplicate to PCA plates. The plates were incubated (30 ºC) for 24 h before CFU counts.

4.2.2. Biofilm formation on stainless steel and polystyrene coupons in a bioreactor

Tests were also performed using a 2 L glass continuous reactor at 25 ºC, aerated and magnetically agitated (Figures 14 and 15). Bacteria (500 mL) from an overnight culture were added ($O.D._{600\ nm}$ = 0.940) to 1.5 L of 0.85% saline solution. The feeding process began 2 hours after the inoculation. The reactor was continuously fed with 0.2 L/h of a sterile solution with 55 mg/L glucose, 25 mg/L peptone, 12.5 mg/L yeast extract, 1.88 g/L monopotassium phosphate and 2.6 g/L sodium phosphate dibasic. Sixteen slides (2.0 cm × 2.0 cm × 0.1 cm) were placed vertically in contact with the bacterial suspension for 5 days, eight made of stainless steel (SS) AISI 316 and eight made of polystyrene. After the biofilm formation period, the slides were transferred to closed sterile flasks where the following conditions were tested in duplicate: cell control with 150 mL of 0.85% saline solution, pH control with 150 mL of 0.12 M citric buffer solution with a pH value at 3 and Fenton reaction with 150 mL of acid citric buffer solution with 20 mM H_2O_2 and 20 mM $FeSO_4.H_2O$ (final concentrations). The flasks were placed in an orbital plate at 25 ºC, for 3 hours. Afterwards, the slides were placed in 10 mL test tubes of saline solution, biofilm was scraped off and vortexed for 2 minutes and the necessary dilutions were performed to determine the numbers of CFU.

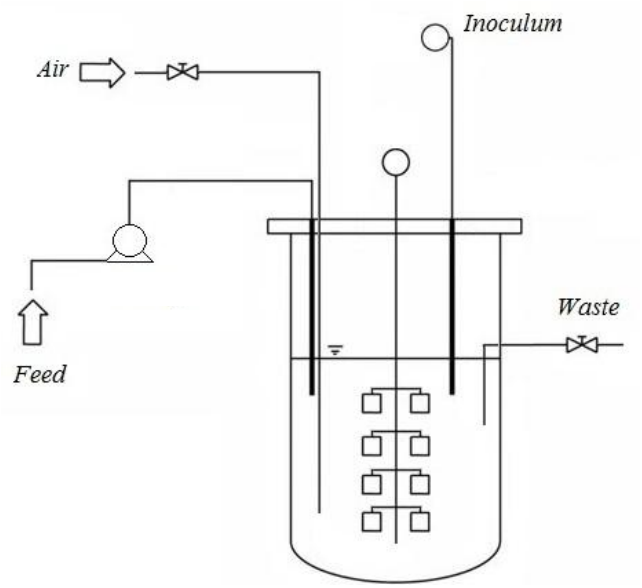

Figure 14. Schematic representation of the experimental setup used for biofilm formation on SS and polystyrene coupons.

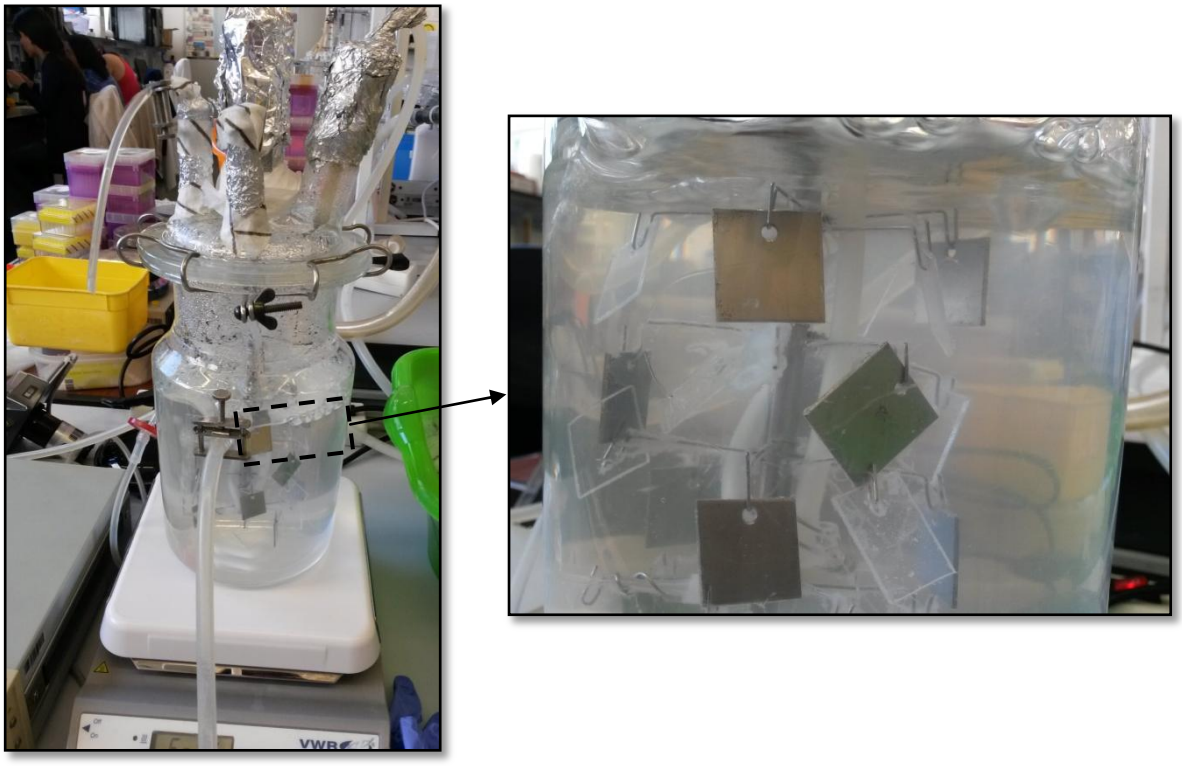

Figure 15. Experimental setup of the reactor used for biofilm formation and a close-up view of the arrangement of SS and polystyrene coupons inside the vessel.

4.2.3. Determination of total volatile solids (TVS)

The dry mass of the biofilms was assessed by the determination of the total volatile solids (TVS) of the homogenised biofilm suspensions, according to standard methods (American Public Health Association [APHA], American Water Works Association [AWWA], Water Pollution Control Federation [WPCF]) (APHA/AWWA/WPCF 1989). Following this methodology, the TVS assessed at 550 ± 5 ºC in a furnace (Lenton thermal designs, UK) for 2 h is equivalent to the amount of biological mass. The biofilm mass accumulated was expressed in mg of biofilm per cm^2 of surface area of the coupon ($mg_{biofilm}/cm^2$).

4.2.4. Statistical Analysis

Statistical analysis was performed as described in Section 3.2.4.

4.3. Results and discussion

4.3.1. Evaluation of the reduction of 24 h-old biofilms

In order to evaluate the disinfection potential of Fenton reagent applied to *E. coli* biofilms, microtiter plates were used as bioreactors to growth 24 h-old biofilms. Previous conducted planktonic state assays allowed the optimization of Fenton reaction, showing that the most effective condition causing cell death was the combination of a citric acid buffer solution at pH = 3 with a Fe^{2+}:H_2O_2 molar ratio of 2:1 (20 mM:10 mM) – chapter 3. However, since biofilms are reported as being more resistant than cells in suspension (Mah et al. 2003), the concentration of H_2O_2 was increased and the molar ratio of Fe^{2+}:H_2O_2 tested was 1:1 (20 mM: 20 mM). The treatment was also performed for 3 hours of reaction time.

According to the results shown in Figure 16, both pH control and Fenton optimized conditions resulted approximately in a 2-log decrease (p<0.05) in the number of viable cells after 3 hours of treatment at pH = 3.

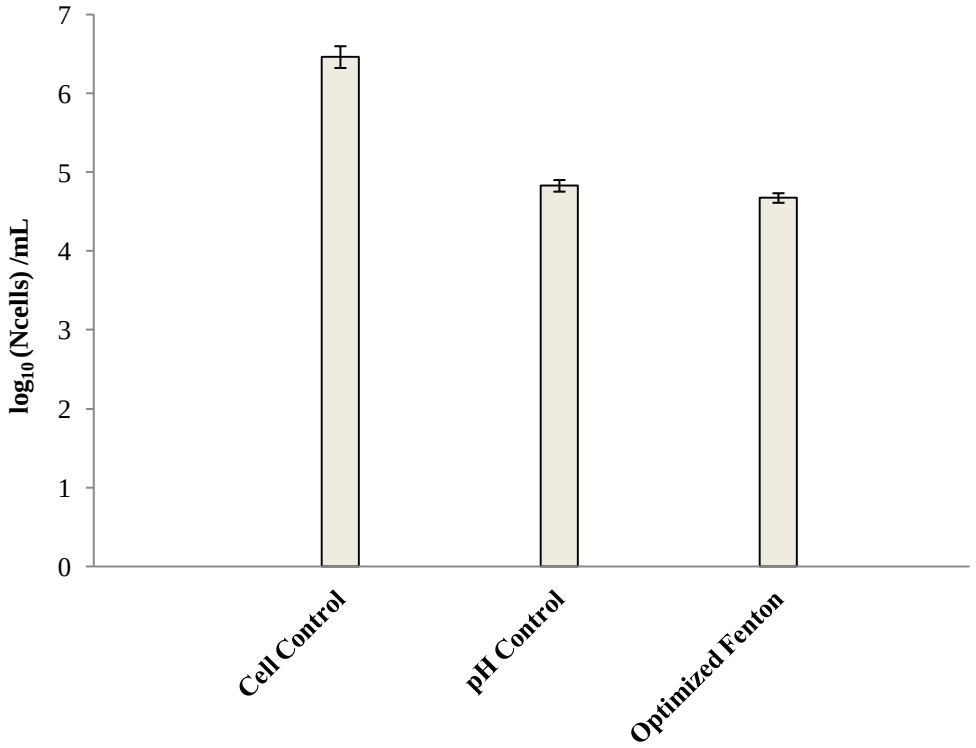

Figure 16. Fenton optimized conditions (citric acid buffer solution at pH = 3 with 20 mM of H_2O_2 and 20 mM of $FeSO_4.H_2O$) applied to 24 h-old *E. coli* biofilms.

This result leads to believe that the bacterial inactivation was mainly caused due to the pH influence. It is not so surprisingly since previous studies stated that low pH values can affect bacterial growth and contribute to cell death (Grandjean et al. 2005).

For the disinfection of autochthonous DW biofilms, Gosselin et al. (2013a) reported that by using a Fenton reaction process with 10 mM of iron sulphate and 15 mM of H_2O_2 at pH = 3, for 1 hour of treatment, resulted in the decrease of the cell number by a factor of 2, the same factor obtained in this study.

In an attempt to achieve higher levels of cell death, the dosage of dissolved iron sulphate was increased from 20 mM to 50 and 100 mM, and the obtained results are presented in Figure 17.

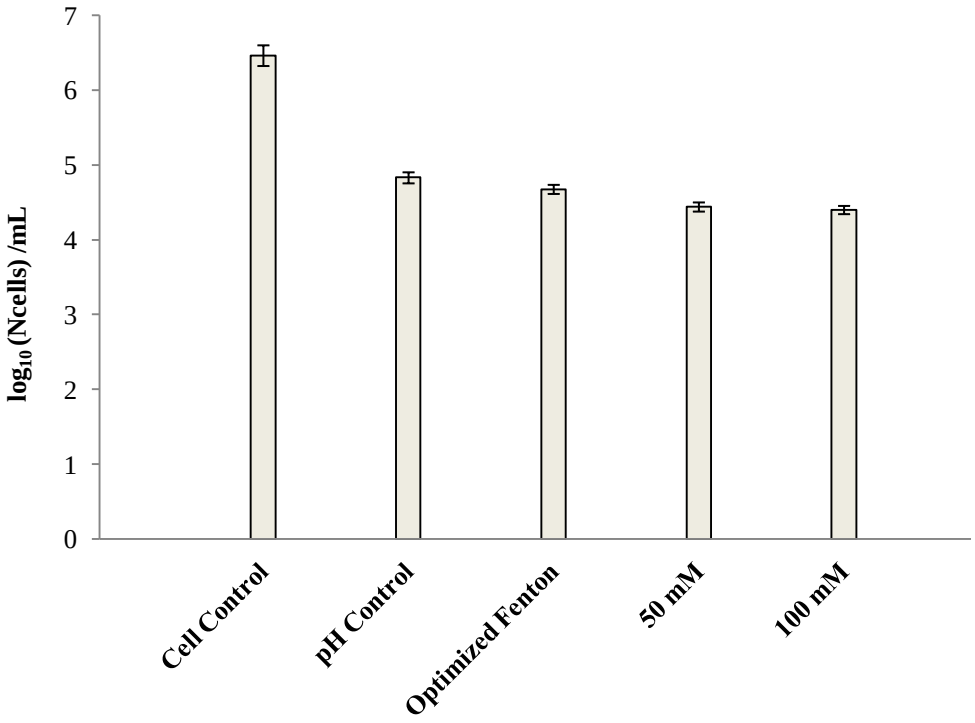

Figure 17. Effect of the increase of iron dosage (50 and 100 mM) in Fenton reaction against 24 h-old *E. coli* biofilms.

Once again, despite the increase of iron dosage in Fenton reactions, the disinfection efficiency did not significantly improved seeing as there is no significant change (p>0.05) between the pH control and the Fenton reagent. Although not having been tested, Gosselin et al. (2013a) stated that the iron catalyst should be available at 100 mM for the reaction to be effective. This study, on the disinfection of 10 months-old biofilms by Fenton reaction at pH = 3 with 15 mM of H_2O_2 for 1 h, showed that even by increasing the iron concentration to 100 mM, the number of inactivated bacteria almost did not increase.

Similarly to the above-mentioned suspension assays, other control solutions were tested in order to study the separate influence of the components on the Fenton reagent: the iron catalyst and the H_2O_2. Figure 18 includes the obtained results for all tested controls and for the optimized Fenton reaction.

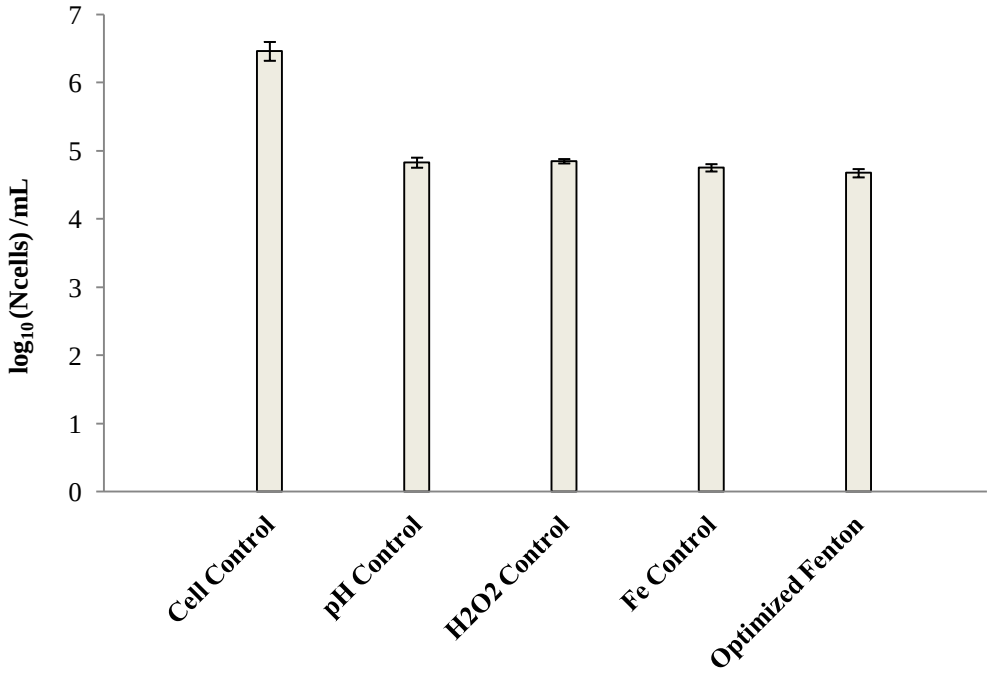

Figure 18. Control solutions (cells, pH, hydrogen peroxide with 20 mM of H_2O_2, iron with 100 mM of $FeSO_4.7H_2O$) and Fenton optimized conditions against 24 h-old biofilms.

Analyzing Figure 18 and in comparison with the cell control, the remaining controls (pH, H_2O_2, iron) and the optimized Fenton reaction also resulted in a 2-log decrease in the number of viable cells of *E. coli* ($p<0.05$). Therefore, the acidic conditions provided by the citric buffer seem to be the main cause to the partial inactivation of the 24 h-old biofilms.

4.3.2. Effects of Fenton optimized conditions in 5 days-old biofilms

The disinfecting potential of Fenton reaction was tested on 5 days-old *E. coli* biofilms grown on stainless steel (SS) coupons, one of the materials found in the pipes of DWDS, and also in polystyrene coupons, the same material of the microtiter plates.

By using the previous tested Fenton reaction conditions (20 mM of H_2O_2 and 20 mM of $FeSO_4.H_2O$), the results of the number of cultivable cells obtained after 3 hours of treatment are shown in Figure 19. Simultaneously, the estimation of the total volatile solids (TVS) for each sample is presented in Table 3.

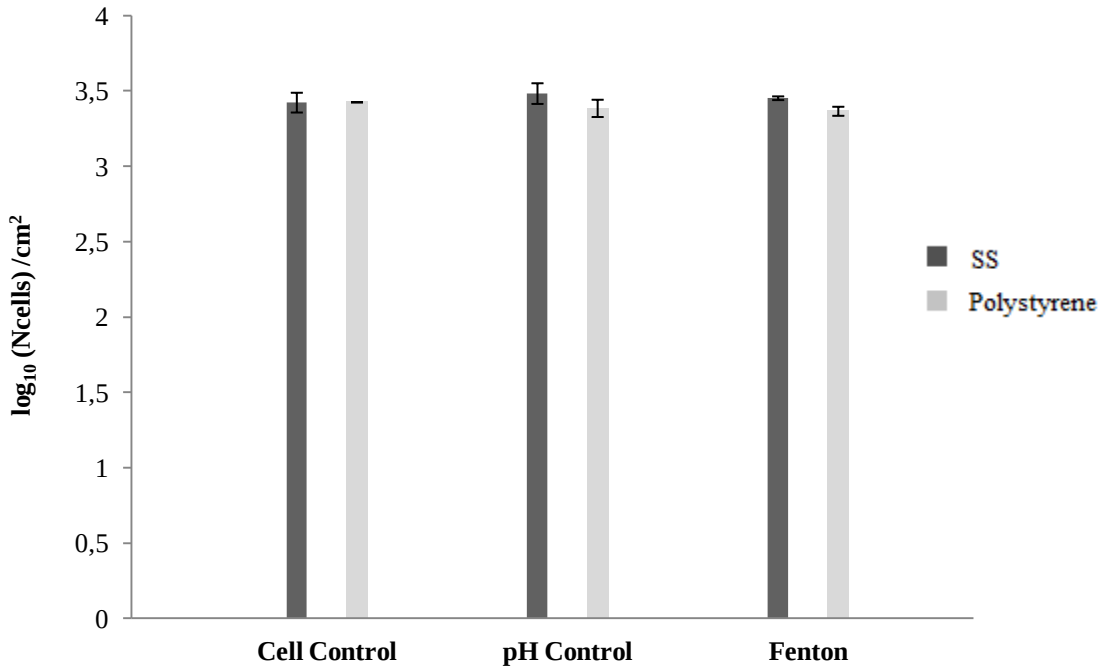

Figure 19. Fenton optimized conditions (citric acid buffer solution at pH = 3 with 20 mM of H_2O_2 and 20 mM of $FeSO_4.H_2O$ for 3 hours) applied to 5 days-old *E. coli* biofilms grown on SS and polystyrene coupons.

According to Figure 19, there is no effect in the number of biofilm CFU neither by using citric buffer at pH = 3, nor by applying the optimized Fenton reaction. In terms of TVS, no significant changes were found with the diverse treatments (p>0.05). This result confirms that the total mass of organic matter present after the Fenton treatment was nearly the same for each sample.

Regarding the type of adhesion surface, SS *vs* polystyrene, no influence was found on bacterial susceptibility to the diverse conditions tested (p>0.05). The numbers of CFU for each condition (cell control, pH control and Fenton reaction) remained approximately the same for biofilms growth on SS and polystyrene coupons.

Table 3. Total volatile solids (mg/cm^2) for biofilm samples grown on SS and polystyrene coupons after exposure to three different conditions (cell control, pH control and optimized Fenton reaction).

TVS (mg/cm^2)	Coupon material	
	Stainless steel	Polystyrene
Cell Control	0.386 ± 0.012	0.239 ± 0.018
pH Control	0.340 ± 0.027	0.244 ± 0.022
Fenton Reaction	0.358 ± 0.020	0.271 ± 0.025

The comparison of the susceptibilities of biofilms formed in the microtiter plates and in the 2 L reactor propose that 5 days-old biofilms were more resistant than those 24 h-old to the diverse treatments.

As previous mentioned in planktonic assays, the induction of an ATR may prevent cell damage in extreme acidic environments. The same can be verified in biofilms (Benjamin and Datta 1995). Welin et al. (2003) reported that *Streptococcus mutans* biofilm cells were significantly more acid resistant than the associated planktonic cells at pH 3. This might be due to the fact that cells experienced acid shocks because of the biofilm's metabolic activity without needing external acidification. Combining the intrinsic ATR with the intercellular signalling and the exchange of genetic material within the biofilm, the result is enhanced resistance. As a result, mature biofilms (5 days-old) can be regarded as more resistant to acidic conditions than recently formed biofilms (24 h-old) (Li et al. 2001).

4.4. Conclusions

Biofilm cells were more resistant to disinfection by Fenton reaction than cells in its planktonic state. The Fenton reaction showed to be almost ineffective in biofilms control under the conditions tested. This is evidenced by the tests with 24 h-old biofilms, where the acidic pH demonstrated to have the major influence in bacterial control. Even by increasing the concentration of the iron catalyst, the efficiency disinfection was almost not improved. Although the acidic pH contributed to bacterial inactivation of 24 h-old biofilms it showed no influence in the control of 5 days-old biofilms. In fact, *E. coli* 5 days-old biofilms were apparently insusceptible to Fenton reaction.

Chapter 5

5. Concluding remarks and future work

5.1. Conclusions

Since most of the traditional disinfection treatments are often ineffective in inactivating pathogen microorganisms from the DW, it is important to understand the disinfection potential of innovate strategies to control the microbial growth in DWDS. Therefore, in this work the antimicrobial potential of Fenton was evaluated against planktonic cells and biofilms of *E. coli*.

- For the optimization of Fenton reaction conditions against suspended cells of *E. coli*, the treatment showed to be more effective at acidic conditions (pH values at 2, 3 and 4), by using higher concentrations of catalyst (dissolved iron sulphate) and hydrogen peroxide and by extending the reaction time up to 3 hours.
- Taking into consideration the selection of sub-lethal conditions, the optimum Fe^{2+}:H_2O_2 molar ratio obtained was 2:1 (20 mM:10 mM) which resulted in a 7-log decrease in the number of CFU cells after 3 hours of treatment at pH = 3.
- Both, exposure to acidic pH and particularly optimized Fenton treatment, significantly interact with the cell surface modifying their physicochemical properties of *E. coli*. Normally regarded as hydrophilic, *E. coli* cells became hydrophobic after exposure to the Fenton reaction and more hydrophilic when the acidic pH was the only variable. Moreover, the Fenton reaction reduced significantly the electron donor parameter of the cell surface.
- Both, acidic pH and Fenton, caused remarkable cell membrane damage as evidenced by the propidium iodine uptake results; the effect is, however, much more noticeable in the presence of Fenton reaction.
- Regarding the biofilm behaviour, *E. coli* 5 days-old biofilms showed to be highly resistant to Fenton reaction followed by 24 h-old biofilms.
- The acidic pH caused the major effects in the disinfection of 24 h-old biofilms, leading to a 2-log CFU reduction.

- Fenton reaction and acidic conditions showed no influence in the bacterial inactivation of 5 days-old biofilms.

Combining all results it is possible to conclude that the Fenton reaction is efficient in the disinfection of suspended cells, moderately effective against young biofilms (24 h-old) and not effective against mature biofilms (5 days-old).

Additionally, while doing this work, the use of Fenton reagent led to a yellow coloration in all the samples, a result of the interaction of the catalyst with hydrogen peroxide. Consequently, it is recommended that the use of homogeneous Fenton processes should be limited to extreme situations in to order to avoid this occurrence, or decrease the iron dose. Alternatively, heterogeneous applications should be envisaged, with the iron immobilized in solid supports or making use of the metals present in DWDS pipes as Fenton catalyst.

5.2. Perspectives for further research

There are several Fenton reaction conditions that have been reported in literature. However, most of them are related to the degradation of toxic chemical pollutants and not in the matter of microbial growth control. In this sense, Fenton reaction mechanisms continue to be an interesting subject for future work.

For further research, more optimizations must be done by testing higher doses of Fenton reagent, extending reaction time in biofilm disinfection, increasing the surrounding temperature of the process (since the reaction is highly dependent upon this parameter) and by using corroded surfaces instead of dissolved iron sulphate, which will allow an increase on the availability/reusability of the catalyst.

The literature also states that the use of chelating agents such as ethylenediamine tetraacetic acid EDTA can enhance the degradation efficiency of iron at neutral pH and the combination of the copper ion with iron leads to a strong increase in the catalytic activity. Therefore, it would also be interesting to test these two hypotheses on microbial growth control.

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Appendix

Figure A1. Example of PCA plate used for counting *E. coli* colony-forming units (CFU) in the range of 20 to 200. Two dilutions were performed in each plate.

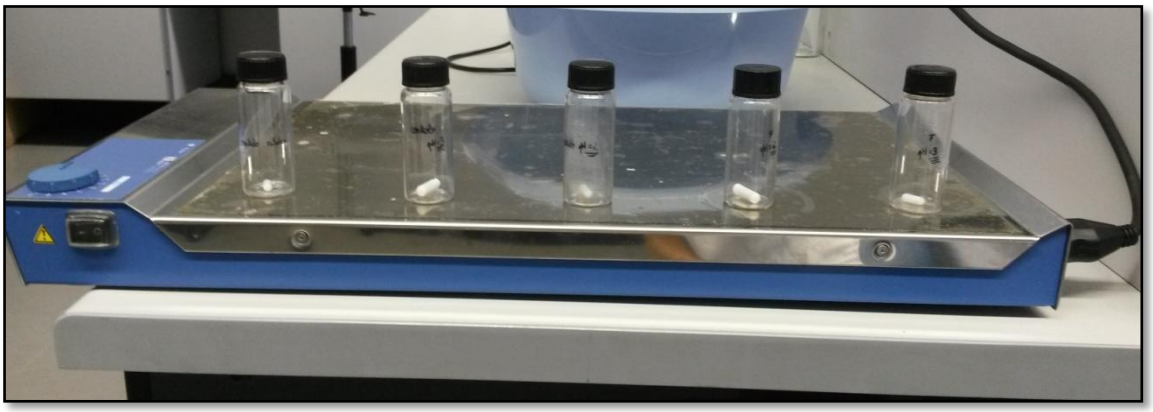

Figure A2. Stir plate with the flasks used to perform cell suspension assays.