Ultrasound enhancement of near-neutral photo-Fenton for effective E. coli inactivation in wastewater

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Abstract

In this study, we attempt for the first time to couple sonication and photo-Fenton for bacterial inactivation of secondary treated effluent. Synthetic wastewater was subjected to sequential high-frequency/low power sonication, followed by mild photo-Fenton treatment, under a solar simulator. It was followed by the assessment of the contribution of each component of the process (Fenton, US, hv) towards the removal rate and the long-term survival; sunlight greatly improved the treatment efficiency, with the coupled process being the only one to yield total inactivation within the 4-h period of treatment. The short-term beneficial disinfecting action of US and its detrimental effect on bacterial survival in long term, as well as the impact of light addition were also revealed. Finally, an investigation on the operational parameters of the process was performed, to investigate possible improvement and/or limitations of the coupled treatment; 3 levels of each parameter involved (hydraulic, environmental, US and Fenton) were tested. Only H_2O_2 increased improved the process significantly, but the action mode of the joint process indicated potential cost-effective solutions towards the implementation of this method.

Keywords: wastewater disinfection, photo-Fenton, high-frequency ultrasound, E. coli, inactivation mechanism

1. INTRODUCTION

Advanced Oxidation Processes (AOPs) have been in the spotlight for more than three decades, as part of a global effort to modernize actual methods of water disinfection. Their action is based on the production of the extremely oxidizing hydroxyl radical ($^{\bullet}OH$) [1], which can attack the chemical structure of the microorganisms' cell wall and inactivate them [2]. Ultrasound has been extensively studied as an AOP, targeting microorganism inactivation, such as bacteria, viruses etc., by either low (~20 kHz) or high frequencies (200+ kHz) [2, 3, 4 and 5]. This method is exploiting the direct mechanical action of the cavitation bubble implosion (low frequencies) as well as the additional production of H_2O_2 and $^{\bullet}OH$ radicals during cavitation (high frequencies); the propagation of ultrasound waves in the aqueous medium initiates the aforementioned actions, by the generation of extreme temperature and pressure conditions [6], which have a proven bactericidal effect [7, 8, 9, and 10].

As far as the ultrasound set-up is concerned, the frequency of the ultrasonic waves is a crucial parameter, for it defines the size of the cavitation bubbles [11]. Literature suggests that the average cavity size is proportional to the acoustic power and inversely proportional to the ultrasound frequency [12]. It is also verified that apart from low frequency/high power ultrasound systems [13, 14], high frequency/low power processes have been proven to efficiently inactivate microorganisms [8, 15, and 16]. However, ultrasound already requires high intensities to achieve total inactivation of microorganisms, and therefore, is considered an expensive application for large volumes of water [2]. Considering all the above, it should be used preferably as a complementary disinfecting method [2].

The photo-Fenton process [17] could play the role of the main disinfecting method, as one of the most efficient methods of hydroxyl radical production [18]. Lately, it has even been used to disinfect drinking water, being a good alternative to chlorination, with its known disinfection by-products formation [19]. However, wastewater is a complex matrix in which many organic and inorganic compounds coexist, such as nutrients, salts and many substances that could influence the outcome of the application of either process. It has been reported that the presence of hydroxyl radical scavengers, namely the organic matter, presents an additional oxidation target and renders AOPs sensitive to the treatment of wastewater [20, 21]. Suppression of these scavengers revealed their importance [10] and also, for years the Fenton reaction was believed to be a pH-restricted reaction in highly acidic regions; it was considered impossible to apply such methods, in matrices with near-neutral pH [22]. However, recent advances [18, 23 and 24] have proven its effectiveness in the neutral area, and in the simultaneous presence of organic matter [18, 25]. Previous work in our group has shown, there is no need for acidification prior to the treatment to keep a significant part of the iron soluble; apart from the direct complexion with bacteria, there are some strong photoactive Fe^{3+} complexes formed in presence of organic matter [21, 25]:

71 $[R\text{-}COO^{-} - Fe^{3+}]^{2+} \rightarrow hv \rightarrow Fe^{2+} + CO_2 + R^{\bullet}$ (1)

72 The cycle continues with the reaction of the regenerated iron with hydrogen peroxide to produce more
73 hydroxyl radicals etc.

74 $Fe^{2+} + H_2O_2 \rightarrow OH^{-} + {}^{\bullet}OH + Fe^{3+}$ (2)

75 In order to increase the amount of water treated by solar-assisted methods, compound parabolic
76 collector reactors have been used [17, 18, 23 and 26], and solar photo-Fenton even was a subject under
77 question, because of the intermittent action of the light [27]. There is a technical issue to be addressed
78 in the intermittent nature of this treatment method, and the existence of "dead" time among the
79 experiment. Typically, a CPC photo-reactor consists of the illuminated surface and the storage-
80 recirculation tank. The recirculating flow of these reactors creates a gap in the illumination for as long
81 as water is present in the (dark) storage tank, allowing bacterial defense mechanisms to deploy [28].
82 Literature indicates a variety of light-to-dark distributions (Table 1), which materialize this difference
83 [17, 18, 23, 26, 29, 30 and 31].

84 Therefore, keeping in mind the improvement of the near-neutral photo-Fenton disinfection while
85 working within realistic operational parameters, for the first time we study the joint ultrasound/photo-
86 Fenton treatment for wastewater, in a CPC-like, lab-scale system. In this manner, we will take
87 advantage of two factors that could work complementing each other: firstly, the exploitation of the
88 dark intervals for sonication, along with the utilization of solar energy for the promotion of a mild
89 photo-Fenton reaction and secondly, the supplementary action these processes have, since, for
90 instance, US can produce H_2O_2 and subsequently, could fuel the photo-Fenton process. In our study,
91 synthetic secondary effluent was used, spiked with *E. coli* K12, recirculating around a sonicated dark
92 reactor and an illuminated batch reactor, under solar simulated light. We aim to:

93 i) Explore the effects of the photo-Fenton factors (light, reactants) and the ultrasonic action
94 (US) on both short and long-term disinfection events; clarification of the effects is
95 attempted by stepwise insertion of the participating actions.
96 ii) Investigate the involved operational parameters (recirculation speed, temperature, light
97 intensity, treated volume and distribution of volumes, iron and hydrogen peroxide content,
98 ultrasound intensity) in a small-scale set-up.

99

100 2. MATERIALS AND METHODS

101

102 2.1. Synthetic secondary effluent preparation

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2.1.1. Microbial methods

The *E. coli* strain K12 (MG1655) employed was provided by the "Deutsche Sammlung von Mikroorganismen und Zellkulturen". Luria-Bertani broth was inoculated with a colony from bacterial *E. coli* pre-cultures, placed in 50 ml plastic falcons for 8 h and then loop inoculated, after 1% dilution overnight (180 rpm and 37°C for 15 h), to achieve stationary phase cells.

Harvested cells were centrifuged and washed three times (5000 rpm, 15 and 5 min for separation and washing, respectively), followed by reservation in saline solution (neutral pH solution with 8 g/L NaCl and 0.8 g/L KCl); a solution of 10^9 CFU/mL is achieved.

2.1.2. Synthetic wastewater composition

The preparation of the synthetic wastewater took place as under the directive of SYMAWE [32]. The initial DOC was 100 mg/L (250 mg/L COD). The experiments used a 10% dilution (in distilled water) of the said composition. The dilution performed corresponds to the COD and DOC values encountered in normal secondary effluents. Finally, the pH of the sample was between 6.5-7. 1 mL of the prepared bacterial solution was used to spike the diluted wastewater, thus resulting in an initial bacterial population of 10^6 CFU/mL.

2.2. Reagents and analyses

The wastewater constituents, as well as the Fenton reagents were used as received. Photo-Fenton experiments were carried out employing ferrous sulfate heptahydrate (Fluka Chemika), hydrogen peroxide (35% by weight, Sigma Aldrich), used as received. The dissolved iron (Fe^{2+}, Fe^{3+}) was measured with the ferrozine method [33], using a UV-Vis Lambda 20 spectrophotometer, provided by PerkinElmer, Schwerzenbach, Switzerland. For 1.6 mL of sample 0.2 of ferrozine solution (4.9 mM) was added, followed by 0.2 mL of hydroxylamine hydrochloride solution 10% w/w. Acetate buffer solution was added for a final 4.5-5 pH value. To determine the concentration of hydrogen peroxide in the sample titanium oxysulfate solution was added, also measured with the same spectrophotometer. The pH of each sample was measured with a pH-meter provided by Mettler Toledo (PH/Ion S220, Seven Compact, Mettler Toledo).

2.3. Description of reactors' set-up

The preliminary study was carried out in plain Pyrex glass batch reactors of 65 mL total capacity. In the set-up presented in Figure 1, the configuration permits the sequential treatment of the synthetic

wastewater; US/photo-Fenton treatment was taking place (or vice versa). The same configuration was used in one of our previous works, (used in [29], similar to the set-up used by Mendez-Arriaga et al, 2009 [34]), synthetic wastewater from a cylindrical double-wall glass vessel (400 mL) was pumped by a peristaltic pump through three glass reactors (diameter 3.8 cm, effective irradiation surface 214.8 cm^2), connected in series, of total volume 230 mL. Temperature was regulated by water recirculating around the reactor and connected to a thermostat. The third reactor effluent was recirculated to the original vessel. Normally, water was inserted in the double-wall reactor and pumped into the irradiated part. Therefore, 230 mL of water were always present under illumination, 70 mL in the distribution system and the rest subjected to sonication.

The ultrasonic waves (275 kHz) were emitted from a piezoelectric 4-cm disc, fixed on a Pyrex glass plate adjusted to the bottom of the double-walled reactor. The intensities applied in all experiments were 10, 20 and 40 W. The electric power was the chosen method to calibrate the ultrasonic equipment. The in-series reactors were irradiated by the Suntest apparatus. The Suntest CPS solar light simulator bears a lamp that emits ~0.5% of the photons at wavelengths <300 nm, ~7% between 300 and 400 nm and the rest follow the solar spectrum. The global irradiance values used in this work were 800, 1000 and 1200 W/m^2, while the corresponding UV values were approximately 19.2, 24.7 and 30.2 W/m^2.

2.4. Experimental design

Two sets of experiments were performed. In a first set of 8 experiments, that we call step-wise construction of the joint treatment process, the elements of the US/hv/Fe/H_2O_2 were gradually and accumulatively applied to the wastewater, in order to determine the individual role of each factor and to detect any synergy among them. Table 2 shows the conditions corresponding to each individual treatment factor when applied. Table 3 summarizes the four subsets of experiments in the step-wise design.

In a second set of experiments (improvement of the process efficiency), eight different variables were individually modified at three levels, while keeping the other variables constant, in order to obtain improved working levels for each variable. Table 4 displays the three values (levels) essayed for each variable. In each experiment the remaining parameters were kept constant and set to the central value shown in the table.

2.5. Bacterial enumeration and regrowth tests

The disinfection efficiency was measured by viable plate counts on Petri dishes containing PCA agar (plastic, 9-cm diameter). The pour-plating method was used and dilutions were made to ensure countable numbers on the plates, i.e. 20-100 colonies/plate. Experiments were performed twice and plating took place in 2-3 consequent dilutions and in duplicates.

Regrowth of bacteria was estimated after the storage of the samples at ambient temperature for 24 and 48 h after the sampling time. Samples were kept in 1.5 mL plastic Eppendorf caps in the dark and the population was measured to assess the post-irradiation events, after their removal from the experimental set-up.

3. RESULTS AND DISCUSSION

3.1. Results of the step-wise construction of the joint treatment process

As far as a potential application of mild photo-Fenton assisted by high frequency/low power ultrasound is concerned, moderate concentrations of reactants are suggested for the evolution of our study, after an initial investigation (data not shown). At 1000 W/m^2 light intensity, an addition of 1 ppm iron and 10 ppm of H_2O_2 will be used, as marginal values of Fenton reagents and 20 W of US power.

3.1.1. Disinfection efficiency

i) Experiments: 1-2 (WW and WW/Fe/H_2O_2).

Figure 2a presents the results of the first part of the experiments, where neither light nor US was applied. Wastewater was recirculated around the non-illuminated, non-sonicated experimental set-up and the corresponding graphs describe the changes when H_2O_2 and iron were added to the solution. We notice the increase of the population, when no reactants were added, due to the existence of nutrients and salts that favor bacterial growth in this water matrix [35]. H_2O_2 is a substance with disinfecting action, while iron itself is not toxic for bacteria. The addition of both reactants causes the initiation of the Fenton reaction, which has a slow, but existing disinfecting ability and within a timeframe of 4 h, we observe a 24.4% reduction in the initial population.

ii) Experiments: 3-4 (US and US/Fe/H_2O_2).

Figure 2b demonstrates the effects sonication has on samples, alongside with the stepwise insertion of the Fenton reagents. The sample recirculates around the ultrasound vessel and the non-illuminated area, being subject to intermittent high-frequency, low intensity sonication. When ultrasound alone is applied, there is a decrease in total bacterial numbers, approaching 27.9%. The concurrent addition of both Fenton reactants (H_2O_2 and Fe^{2+}) in the sonicated sample causes an 82.1% reduction in the bacterial population, compared to 27.9 % reduction for US treatment and 24.4% for Fenton treatment alone. This indicates a synergy between sonication and the Fenton reagents; a synergy factor of 1.57 is demonstrated by the disinfecting efficiency of the reactions.

During sonication, the breakage of the cavitation bubbles can lead to the formation of an almost point-sized heat source [36, 37], with local temperatures approaching 2000 K and pressures of 200 atm. These extreme conditions can cause lysis of water molecules and along with that, extra production of hydroxyl radicals [8]. The presence of the afore-mentioned particles in real wastewater and the bacteria (in our matrix) could also play another important role, since the collapse of the cavitation bubbles near a particle in the medium could cause micro-jets, depending on the size of the particle [38] and could also form "weak spots" in the body of the liquid; these are potential places to form a cavity [39]. It has been also reported that the presence of some salts causes a baro-protective effect on the cells [40] and samples with higher contents of soluble solids would require higher sonication times. Apart from the physical damage, during the ultrasound treatment of the sample, there is ample generation of reactive oxygen species ($^{\bullet}OH$ radicals [41], singlet oxygen [42, 43]), as mentioned before, which are known to stress bacteria and lead to cell death [25, 44].

Finally, the addition of peptone (present in the synthetic wastewater) and the generally, presence of nitrogen compounds has been reported to delay the sonicated degradation of phenols [45]. However, nitrogen, under the presence of ultrasound waves can form NOx (nitrate and nitrite). Its reaction with singlet oxygen (as produced before) [43] produces peroxynitrite ($ONOO^-$) [46]. Peroxynitrite is included in the reactive nitrogen species and can cause significant injures to various structures of the cell (free radical damage or attack against the respiratory chain) [46].

The synergistic action of US and Fenton processes can be attributed to the exploitation of the recombined H_2O_2 (from $^{\bullet}OH$), which is less oxidative than the hydroxyl radical itself, and with that, the re-initiation of the Fenton reaction with new reactants. Also, the ultrasound process, according to Kryszczuk [47], increases the transient breakage of the bonds among the molecular components of the cell membrane, which increases the permeability of the cell in external substances [48]. Therefore, the introduction of Fe^{2+} in the cell is easier and its presence inside the cell can produce hydroxyl radicals very close to vital functions of the cell, as well as the DNA [25] due to the induced internal Fenton process.

iii) Experiments: 5-6 (hv and hv/Fe/H_2O_2).

The 3rd set of experiments is dedicated in the investigation of the impact of light in the sequential process. In all experiments light is provided at 1000 W/m^2, but in total, intermittent irradiation is provided to the system; there is an illuminated regime and a non-illuminated one, in the Suntest apparatus and the (inactive) sonication vessel (and tubing). In one of our previous works [29], we demonstrated the impact light intermittence has on bacterial disinfection and survival, while continuous supply or very fast recirculation around illuminated and dark regimes favors disinfection, with the same set-up. Therefore, photo-Fenton is promoted in non-intermittent regimes or, as in our case, short dark interval periods.

As it can be seen from Figure 2c, light, even in non-continuous form, is very effective and results in high inactivation rates. Its disinfecting action is dominating the removal process, until the Fenton reagents are present, and solar-assisted photo-Fenton is induced. The action of photo-Fenton is taking place within the Suntest and dark (normal) Fenton takes place during the rest of the time, in a 0.85:1 time distribution (46% photo-Fenton over 54% Fenton). After an initial delay, which is demonstrated as a shoulder in the graph, reaction is more effective by the hv/Fe/H_2O_2 than the corresponding solar treatment.

Spuhler et al. [25] have reviewed the mechanism of bacterial inactivation by the photo-Fenton reaction in near-neutral water with organic components, and have suggested the possible sources of ROS production and cellular photo-oxidative damage, as well as the damage done by the ROS themselves, deriving from the photo-Fenton reaction. In our suggested treatment method, these mechanisms are completely compatible, explaining the majority of the actions and other works on near-neutral photo-Fenton mechanisms describe fully the mechanisms, so we will not further analyze their findings.

iv) Experiments: 7-8 (hv/US and hv/US/Fe/H_2O_2).

The final group of experiments are presented in Figure 2d. This graph summarizes the results of the joint treatment by light and ultrasound. It is clear, after a comparison with Figure 2c, that when light is present, its disinfecting action is dominating the process. However, we observe that the only case total disinfection is achieved, is by the sequential US/pF system. In this system, wastewater spends its time distributed 46% under photo-Fenton, 14% in the dark (dark-Fenton) and 40% in the sonication vessel (US/Fenton). The experimental time has less inactive periods, and we observe that it has a significant impact in the total inactivation of the bacterial populations in less than 4h. Here, the photo-Fenton/US synergy is low in terms of bacterial counts, efficiency was improved in a relatively low percentage, but only the coupled process resulted in total disinfection in 4 h. The elevated efficiency and total inactivation for the first time, is attributed to the combination of all the previous actions (in US and/or light), as well as the following actions (a graphical summary of all the actions is presented in Figure 3):

i. The hydroxyl radical is a short living ROS, and it occurs not to reach the target in all cases and often recombines to create H_2O_2 [49]. Therefore, the addition of ultrasound directly produces hydroxyl radicals and H_2O_2; the $^{\bullet}OH$ directly attacks the cell and H_2O_2 participates in the photo-Fenton reaction (2). Alongside with the added H_2O_2, there is additional production, fueling the Fenton reaction and thus, improving the overall efficiency of the treatment.

ii. As we described before, with ultrasound waves, the loosening of transient bonds and insertion of Fe^{2+} in the cell is increased, which promotes the internal Fenton reaction. After the completion of the Fenton reaction, light reduces Fe^{3+} to Fe^{2+}, and re-initiates a radical production inside the cell (internal photo-Fenton).

iii. Low frequency ultrasound has been proven [50] to reduce Fe^{3+} in the form of ferrous ions (Fe^{2+}). The average size of the bubble however decreases when frequency is increased, in our system [12]; nevertheless, cavitation still takes place. Therefore, it is possible that an action like this could provide an additional source of iron available for the photo-Fenton process, and progress the regeneration of the catalyst in the (otherwise) non-illuminated part of the time. In that way, more available ferrous ions can be present in the solution.

iv. We mentioned the extreme temperature and pressure conditions that take place during the collapse of the cavitation bubbles. The interior part of the bubble, under these conditions, is known to emit light, under the phenomenon of sono-luminescence [8]. The optical aspects of this phenomenon have been studied [51] and the emitted light wavelengths fall into the necessary ones possibly able i) to induce the regeneration of the photo-Fenton reaction catalyst, ii) inflict direct UV damage to the cell. However, the necessary energy to achieve this is still under question.

v. Apart from the radicals' production through the normal photo-Fenton cycle, the presence of light is participating in another series of reactions with nitrogen compounds. The photolysis of nitrate and nitrites (produced by the participation of the US in the process) can lead to additional hydroxyl radical production [45]:

Photolysis of Nitrate:

$$NO_3^- + h\nu \rightarrow NO_2^- + \frac{1}{2}O_2 \quad (3)$$

$$NO_3{}^- + h\nu \rightarrow NO_2\boldsymbol{\cdot} + O^{\bullet -} \quad (4)$$

$$O^{\bullet -} + H_2O \leftrightarrow HO^{\bullet} + OH^- \quad (5)$$

Photolysis of Nitrite:

$$NO_2^- + h\nu \rightarrow NO^{\bullet} + O^{\bullet -} \quad (6)$$

$$O^{\bullet -} + H_2O \leftrightarrow HO^{\bullet} + OH^- \quad (7)$$

3.1.2. Post-processing events: long-term disinfecting activity of the joint process

The monitoring of the bacterial population for 48 h after the completion of the experiment has indicated some rather interesting aspects on the characteristics of the driving forces of the joint disinfection process. Figure 3e demonstrates the post-treatment bacterial counts, starting from the moment the reactions have stopped (4-h mark). There are two big groups of charts we can distinguish, which present different behavior: the non-irradiated and the irradiated processes.

First of all, it is observed that if there is no light or US treatment involved, as expected, bacterial populations tend to increase their numbers. The presence of the Fenton reagents inflicts a constant, but relatively slow, elimination of the present bacteria. The bacterial population in the sonicated samples (square traces), although having survived the 4 hours of sonication, is significantly lowered in the days following treatment. This observation seems to suggest some type of permanent damage that has affected their cultivability. Even more, the US/Fenton treatment has shown that even with high remaining bacterial values, after 2 days, the damaged bacteria have almost completely succumbed, due to the combined sonication damage and the Fenton reaction that was still ongoing.

The long term effects caused by the sonication of the water sample can be summarized as follows: The employed acoustic range promotes the production of hydrogen peroxide, which is an indicator of the formation of other oxidative species [8]. In this frequency, the generated hydroxyl radicals are easily transferred away from the bubbles [7, 52, 53 and 54]. The high-frequency damages include dislocation of the cell membrane, which often leads to intracellular content leakage, due to the disruption of the cell wall integrity [55, 56]. As a result, bacterial viability is lost; a gradual degradation of the cell membrane takes place due to the attacks of the hydroxyl radicals in the medium and vital parts of the bacterium are attacked [55], reduced potassium uptake and restricted DNA and protein synthesis have also been reported [57]. Also, programmed cell death and cell apoptosis were also recently mentioned [16, 58], which explain the delayed inactivation of cells. These processes can explain the behavior of the sonicated samples for the 48-h monitoring period. Finally, in the combined process before, we mentioned the greater iron uptake by the cells due to its transformation [47, 48]. We believe that this process is of high significance, supported by the post irradiation events; within 48 h there are no more cultivable bacteria, and comparing with the plain dark Fenton process, we attribute the change in the already apoptotic cells, which are easier to succumb to further oxidative damage after their sonication and finally to the increase of the internal Fenton process.

The second group of experiments, where light treatment was involved, all demonstrate zero counts within two days. Light has significantly impaired bacterial reproduction and all samples that were irradiated lead to total inactivation. Photo-Fenton treatment has proven to completely inactivate in less time (<24 h), totally eradicating the small bacterial counts left during the treatment. Total inactivation can however be reached within 24 h under joint US/solar light treatment even in the absence of iron, due to the sequential damaging by US and solar light. No regrowth was observed in any of the

experiments within a 48h period. In addition to that, the coupled US/pF process, has maintained its zero count throughout the 2-day period, with no noticeable regrowth or recovery.

3.2. Improvement of the process efficiency: Investigation of the operational parameters involved in the US/pF coupling

Having observed the total and permanent inactivation for the solar-assisted US/pF system, it is our interest to examine how other types of operational parameters could influence the process efficiency. Possibilities for improving the process and investigating its flexibility and robustness are ample. The parameters to be studied are summarized in Table 4, and divided in hydraulic, environmental and chemical (US and Fenton factors).

3.2.1. Hydraulic parameters

Figures 4a-c present the investigation that has been conducted, to study the effects of the modification of the recirculation rate, the number of in-series illuminated reactors, and the treated volume, respectively. The three recirculation rates correspond to 1.87, 3.44 and 4.39 L/h. Hulsmans et al. [59] suggest that increasing the flow rate in a US system resulted in higher disinfection rate. However, in our system, changing the recirculation rate causes the faster sequential change from US to photo-Fenton, and therefore, shorter cycles of treatment. This leads to more completed rounds of sonication/Fenton, and consequently, better treatment results.

Figure 4a indicates the said effect; the explanation lies within the nature of the two actions. On the one hand, sonication assists the inactivation and the transformation of iron more times, so photo-Fenton is more efficient, and on the other hand, more completed cycles of illumination, provide higher possibilities for the emitted photons to target the bacteria (direct action) or the production of hydroxyl radicals to attack them (indirect action). Table 5 includes the hydraulic calculations concerning the timeframe of the actions.

Furthermore, in Table 5 we notice the effect of changing the number of available reactors for the photo-Fenton treatment. Reducing the number of reactors affects:

i) The available illuminated volume: The photo-Fenton action is also reduced, because less volume is under illumination (reduction of both direct and indirect damage).

ii) The volume of the sonicated water: Since the volume remains 500 mL and the reactors of ~75 mL are reduced, more water remains in the sonication chamber. Therefore, the ratio of US power/volume of water is modified and less power (but more time) is available for each mL of wastewater.

As a consequence, we observe in Figure 4b that reducing the available reactors from 3 to 1 modifies the photo-Fenton to US treated volume ratio from 1.15 to 0.52 and 0.27, respectively (tubing volumes are neglected, because of the slow rate of the dark Fenton reaction). In terms of bacterial numbers, the inactivation rate is of 6, 4 and 3 $\log_{10}$U, for 3, 2 and 1 reactor available. Although this looks as a diminishing effect on the process efficiency, it also indicates that if less irradiation is available, the process is still effective, and an extension in the time would eventually lead to total inactivation. We believe this is an indication of the assisting/complementary character of the sonication, whenever photo-Fenton is not available.

Finally, although literature suggests that modifying the sonication volume has not a significant effect on the efficiency of the sonication process (if the power-to volume ratio is respected) for bacterial inactivation [59, 60], in Figure 4c we observe 2 and 3 $\log_{10}$U of difference, respectively. A 20% and 40% increase of the volume lead to 33% and 50% reduction of the efficiency. This is explained by the domination of the process by the photo-Fenton reaction, rather than the sonication; it seems that it is not cost-effective to increase the total volume beyond a certain value.

3.2.2. Environmental influence

In our experimental set-up, an investigation of the temperature took place to assess the available operating temperatures of the coupled treatment, summarized in Figures 5 a-b. The first operational limit was the temperature of 30˚C, to protect the piezoelectric disc. Recirculation of refrigerated water around the mantle of the US vessel ensured that the temperature was maintained within this limit. Reducing the operational temperature lead to decrease of the inactivation efficiency; the reaction became slower and less effective. On the one hand, this behavior (Figure 5a) is attributed to the reduced kinetics of the photo-Fenton reaction: it is known that temperature increases chemical reactions' kinetics, plus Ortega-Gomez et al [61] revealed the reduced inactivation rate in another bacterial species but also mesophilic with similar optimal growth temperature with *E. coli*, according to the Arrhenius equation and on the other hand, treating wastewater at temperatures close to the optimal growth conditions, can delay bacterial inactivation [62].

On the contrary, altering the irradiation intensity (Figure 5b) did not significantly affect the efficiency of the process. It can be seen that ±20% difference in intensity resulted in similar required inactivation times. The initial reaction kinetics is faster at 1200 W/m^2 because of the increased direct action of the light; higher intensities lead to faster bacterial inactivation rates [63]. The process is nevertheless effective even for lower intensities, suggesting that disinfection is possible even in days of low solar radiation.

3.2.3. Fenton and sonication factors

Figures 6a-c present the results of the investigation over the constituents of the Fenton reaction, as well as the only modifiable parameter of the US, the sonication power. We observe from figures 6a and 6b, that there is a minimum quantity of the Fenton reagent required to be initially present, in order to maintain the integrity of the reaction throughout the treatment time. For instance, when the initial H_2O_2 concentration was reduced to 5 ppm, after the 2nd hour the reaction kinetics modified and inactivation rate was impaired. The oxidation of organic matter by the hydroxyl radicals is competing against the bacterial inactivation [21]. The contribution of photo-Fenton is reduced and the reaction continues with the produced H_2O_2 and the direct effects of irradiation and US. However, doubling the initial concentration of H_2O_2, provides enough $^{\cdot}OH$ radicals, to achieve the fastest inactivation time in all our experiments. Although unique, this case suggests a doubling on the supply costs of the process, but at the same time, a chance to improve otherwise impaired inactivation rates observed in previous cases.

Same effects apply for the iron content of the initial sample. When the iron concentration was halved, reaction rate and final outcome was mitigated, compared to the normal processes. Even though the US indirect action benefits iron transformation to the more useful state of ferrous ions, as a catalyst, it is obvious that it is in shortage. As soon as the initial concentration was doubled, no significant effect was observed, probably due to the saturation of the sample although presence of organic matter sustains iron in solution [21]. Hydroxyl radical production reached its peak and therefore no improvement was observed in bacterial inactivation.

Finally, the modification of the acoustic power was investigated, and its effects on removal efficiency are demonstrated in Figure 6c. Increasing US power results in higher particle breakage [64] and more efficient removal, in the high frequencies [59, 60]. In our system, decreasing the power from 20 to 10 W, and consequently, the power-to-volume ratio, decreased the efficiency, although in a non-linear, cost-effective manner; 50% reduction did not result to 50% decrease of the inactivation, but to 33%, although the main target is total inactivation. This suggests that the process can operate in economically low power, increasing its feasibility in real-scale application, and proves the complementary character of the two processes. Increasing the power to 40 W did not really enhance the removal efficiency, probably because 20 W was enough to induce the effects of sonication in the sample or the increase was not high enough to demonstrate measurable change, in the hourly sample scale.

3.3. Operational cost and full-scale application considerations

The proposed hybrid treatment has already been proven efficient against the treatment of pollutants in bench scale [49] and the results found so far support its efficiency against *E. coli*. However, the

current set-up employs almost 40 kW/m^3 electrical energy per hour, and compared with other low-frequency sonication applications (e.g. [59] or [65]), the electrical energy required is much higher, in a 4-h scale. The sum drops if a higher amount is treated, but is still economically challenging. The main problem is located in the drawback high frequency sonication engulfs, that requires long residence times in order to achieve total disinfection, even with the aid of photo-Fenton. In order to render this solution economically competitive some other factors need to be taken into account.

First of all, it was observed that high frequency disinfection did not dramatically increase disinfection rates directly, but a long term inactivating effect was demonstrated. Sonicated samples after 48 h presented lower bacterial counts, and null counts when the Fenton reagents were present. These results indicate the possibility to reduce the residence times, and further investigate the correlation of sonication with this long term inactivating effect. Hence, depending on the use of the treated water afterwards, the residence time could differ, reducing the direct operational costs.

Furthermore, the investigation of the operational parameters, showed potential pathways to improve the process. During this study, the contribution of each factor was studied separately. It was found that e.g. doubling the base concentration of H_2O_2 (from 10 to 20 ppm) total inactivation was achieved in 3 h, almost 25% reduction in the residence time and increase of the iron concentration from 1 to 2 ppm had no measurable effect, but the estimation of their combined effect is unknown. However, it is normal to expect higher Fenton efficiency with higher reactant concentrations and further reduction of the residence times, as well as improved rates with higher sonication power values. Therefore, a poly-parametric design of experiments for the optimization of the system should be conducted, to define the most economic operational conditions.

Concerning other photo-Fenton applications for pollutants degradation, Klamerth et al [66] used values around 50 and 5 ppm for hydrogen peroxide and iron and Rodriguez-Chueca et al [67] used 5-50 and 2.5-10 ppm, respectively. Switching from mild to normal photo-Fenton values for bacterial inactivation in wastewater will holistically benefit the system efficiency. Apart from the direct photo-Fenton effect which can be enhanced, a positive impact is also expected on the indirect effects of sonication with the interactions with the iron content of the sample and the radical production. Finally, a pilot scale plant, with optimized operational parameters could give a better view in the expected operational costs. However, the marginal values used in this study indicated the promising potentials of the system and suggested that the system has the possibility to be rendered economically feasible, provided that long-term inactivation can be achieved by shorter sonication times.

4. CONCLUSIONS

An initial study concerning the treatment of wastewater microorganisms has been made, by the application of sequential mode high frequency ultrasound and mild photo-Fenton. In the stepwise introduction of treatment factors, light has been, by far, the most significant effect amongst all parameters. Light alone has proven to be much more effective than US, Fenton, or the combination of the two. Also, light combined with Fenton (photo-Fenton) and US with photo-Fenton (US-pF) have been the two most effective disinfection options. This can be attributed to the well-known multi-level effect of light, interpreted by the direct action of the light against bacteria, the indirect ROS production and the direct role of light in Fe^{2+} reduction (photo-Fenton). High frequency-low intensity ultrasound alone has not provided significant immediate bacterial reduction, but in long term, causes either apoptotic behavior or increased susceptibility to the Fenton damage. When combined with light, US has resulted in high inactivation rates in 4 h, and even higher when the Fenton reagents were also introduced (joint US-pF process). This makes US-pF treatments an attractive alternative in permanent (bacteriologically non-recurring) treatment methods.

Regarding the contribution of the operational parameters, temperature and volume introduce important constraints: Temperature favors disinfection but must not exceed 30ºC for US source protection; increasing the sonication volume will result to higher US-to-pF ratios and lower efficiencies. However, modification of the US-to-pF volume ratio can be opted regarding the post-treatment handling of the sample; if immediate disinfecting action is required, pF can be promoted, in any other case the continuous decay US causes will result to total inactivation during sample storage. Also, addition of extra hydrogen peroxide and iron seemed to benefit bacterial inactivation. From the scope of our work, the choice of mild photo-Fenton was satisfactory, but in a real application, this choice, over normal amounts of reagents can be also altered depending on the requirements downstream.

Nevertheless, our results indicate that this US/pF process surpasses limitations that averted installations of either one of the processes on wastewater treatment, such as the dead times in the dark storage tanks, the power-to-volume ratio of the ultrasound, etc. It seems that the combination of the two actions in sequential form helps overcome the disadvantages each method has separately; whenever Fenton was limited, cellular regeneration was hindered by US, thus compensating during dark periods and therefore improving photo-Fenton treatment efficiency. The two actions act complimentarily to each other, with ultrasound providing an additive effect in the photo-Fenton action mode.

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List of Tables

Table 1 – Hydraulic characteristics of previous works in CPC reactors

	Flow rate (L/min)	Total volume (L)	Volume-to-flowrate ratio	Illuminated Volume	Volume in the dark	Light-to-dark ratio
Fernandez-Ibañez et al. (2009)	20	14	0.70	32%	68%	0.47
Moncayo-Lasso et al. (2009)	17.5	20	1.14	45%	55%	0.82
Fernandez-Ibañez et al. (2005) (varied flowrates)	5, 13, 22.5	11	2.2, 0.85, 0.49	49%	51%	0.96
Rincon & Pulgarin (2007) (min, max capacity)	20.5	37, 70	1.80, 3.41	65%, 34%	35%, 66%	1.86, 0.52
Sciacca et al. (2011)	24.2	18	0.74	83%	17%	4.88
Giannakis et al. (2013)	0.03, 0.06, 0.07	0.7	22.58, 12.28, 9.59	33%	67%	0.49
Ndounla et al. (2013)	2	25	12.5	60%	40%	1.53

Table 2 – Parameters involved in the joint treatment process

Factors	Values	Other parameters
Light	1000 W/m^2	***Temperature: 30˚C***
Ultrasound	20 W	***Recirculating Flow rate: 4.39 L/h***
Iron	1 ppm	***Treated Volume: 500 mL***
H_2O_2	10 ppm	***Initial Population: 10^6 CFU/mL***

Table 3 – Subsets of experiments in the step-wise construction of the joint hv/US/Fe/H_2O_2 treatment process.

Experiments	Treatment constituents
1-2	WW and WW/Fe/H_2O_2 - Wastewater with no treatment - Wastewater + Fe/H_2O_2
3-4	US and US/Fe/H_2O_2 - Wastewater+US - Wastewater+US+Fe/H_2O_2
5-6	hv and hv/Fe/H_2O_2 - Light - Light+/Fe/H_2O_2 (photo-Fenton)
7-8	hv/US and hv/US/Fe/H_2O_2 - Light+US - US+photo-Fenton

Table 4 – Overview of the investigation of the operational parameters

Factors[1]	Level 1	Level 2	Level 3
Hydraulic			
Pump rpm	33	66	**99**
No. of Illuminated vessels	1	2	**3**
Wastewater volume (mL)	**500**	600	700
Environmental			
Temperature (˚C)	10	20	**30**
Light Intensity (W/m^2)	800	**1000**	1200
Fenton / Ultrasound			
H_2O_2 Concentration (ppm)	5	**10**	20
Fe Concentration (ppm)	0.5	**1**	2
US Acoustic Power (W)	10	**20**	40

[1]*Central values are annotated with bold.*

708

Increasing recirculation speed from 33 to 99 rpm (1.87 to 4.39 L/h)												
Reactors	3	33	rpm	Reactors	3	66	rpm	Reactors	3	99	rpm	

709 *Table 5 – Hydraulic calculations on the reactor set-up*

710

Volume	**500**	**mL**	**1.87**	**L/h**	**1.87**	**L/h**	**Volume**	**500**	**mL**	**3.44**	**L/h**	**3.44**	**L/h**	**Volume**	**500**	**mL**	**4.39**	**L/h**	**4.39**	**L/h**
Light	230	mL	7.38	min	46	%	**Light**	230	mL	4.01	min	46	%	**Light**	230	mL	3.14	min	46	%
Tubing	70	mL	2.25	min	14	%	**Tubing**	70	mL	1.22	min	14	%	**Tubing**	70	mL	0.96	min	14	%
US	200	mL	6.42	min	40	%	**US**	200	mL	3.49	min	40	%	**US**	200	mL	2.73	min	40	%
Total	**500**	**mL**	**16.04**	**min**	**100**	**%**	**Total**	**500**	**mL**	**8.72**	**min**	**100**	**%**	**Total**	**500**	**mL**	**6.83**	**min**	**100**	**%**
Increasing illuminated volume from 1 reactor to 3 (75 to 230 mL)																						
Reactors	**1**		**99**	**rpm**			**Reactors**	**2**		**99**	**rpm**			**Reactors**	**3**		**99**	**rpm**			
Volume	**500**	**mL**	**4.39**	**L/h**	**4.39**	**L/h**	**Volume**	**500**	**mL**	**4.39**	**L/h**	**4.39**	**L/h**	**Volume**	**500**	**mL**	**4.39**	**L/h**	**4.39**	**L/h**
Light	75	mL	1.03	min	15	%	**Light**	150	mL	2.05	min	30	%	**Light**	230	mL	3.14	min	46	%
Tubing	50	mL	0.68	min	10	%	**Tubing**	60	mL	0.82	min	12	%	**Tubing**	70	mL	0.96	min	14	%
US	375	mL	5.13	min	75	%	**US**	290	mL	3.96	min	58	%	**US**	200	mL	2.73	min	40	%
Total	**500**	**mL**	**6.83**	**min**	**100**	**%**	**Total**	**500**	**mL**	**6.83**	**min**	**100**	**%**	**Total**	**500mL**	**mL**	**6.83**	**min**	**100**	**%**
Increasing total treated volume from 500 to 700 mL																						
Reactors	**3**		**99**	**rpm**			**Reactors**	**3**		**99**	**rpm**			**Reactors**	**3**		**99**	**rpm**			
Volume	**500**	**mL**	**4.39**	**L/h**	**4.39**	**L/h**	**Volume**	**600**	**mL**	**4.39**	**L/h**	**4.39**	**L/h**	**Volume**	**700**	**mL**	**4.39**	**L/h**	**4.39**	**L/h**
Light	230	mL	3.14	min	46	%	**Light**	230	mL	3.14	min	38	%	**Light**	230	mL	3.14	min	33	%
Tubing	70	mL	0.96	min	14	%	**Tubing**	70	mL	0.96	min	12	%	**Tubing**	70	mL	0.96	min	10	%
US	200	mL	2.73	min	40	%	**US**	300	mL	4.10	min	50	%	**US**	400	mL	5.47	min	57	%
Total	**500**	**mL**	**6.83**	**min**	**100**	**%**	**Total**	**600**	**mL**	**8.20**	**min**	**100**	**%**	**Total**	**700**	**mL**	**9.57**	**min**	**100**	**%**

711

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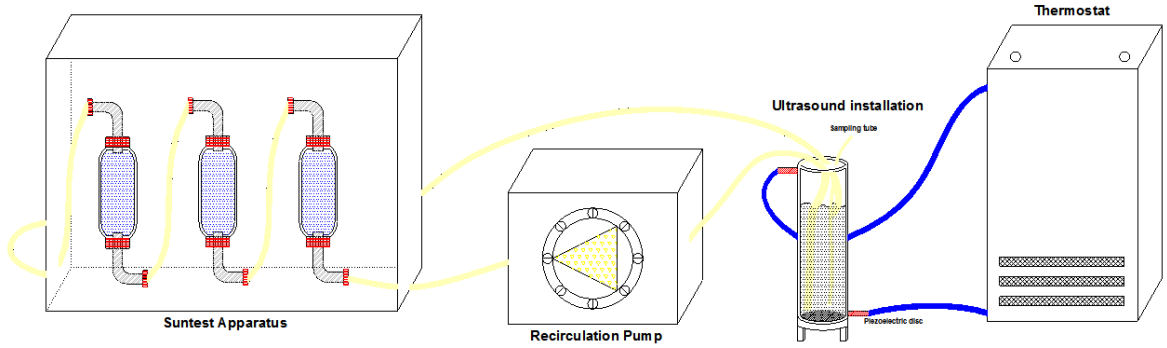
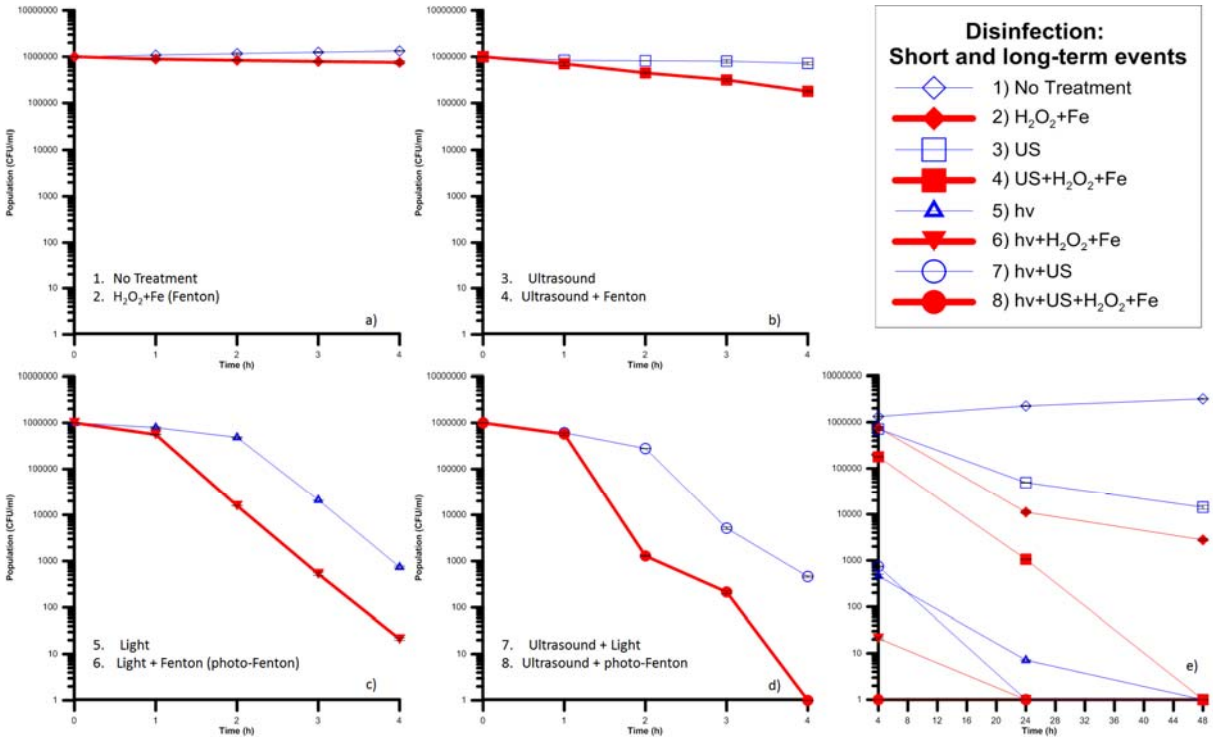


713

714 Figure 1 – Experimental set-up, consisting of the illuminated area (Suntest apparatus), the recirculation pump,
715 the (dark) sonication vessel and the temperature control (thermostat). The flow direction is clockwise, water is
716 introduced at surface level and sampled from the bottom of the vessel.

717



718

719 Figure 2 – Experimental results from the coupling of photo-Fenton and sonication. a) Experiments 1-2 (WW and
720 WW/Fe/H_2O_2), b) Experiments 3-4 (US and US/Fe/H_2O_2), c) Experiments 5-6 (hv and hv/Fe/H_2O_2) and d)
721 Experiments 7-8 (*hv/US and hv/US/Fe/H_2O_2*). e) Long-term inactivation events for 48 h (time axis initiates in the
722 4-h mark, after treatment).

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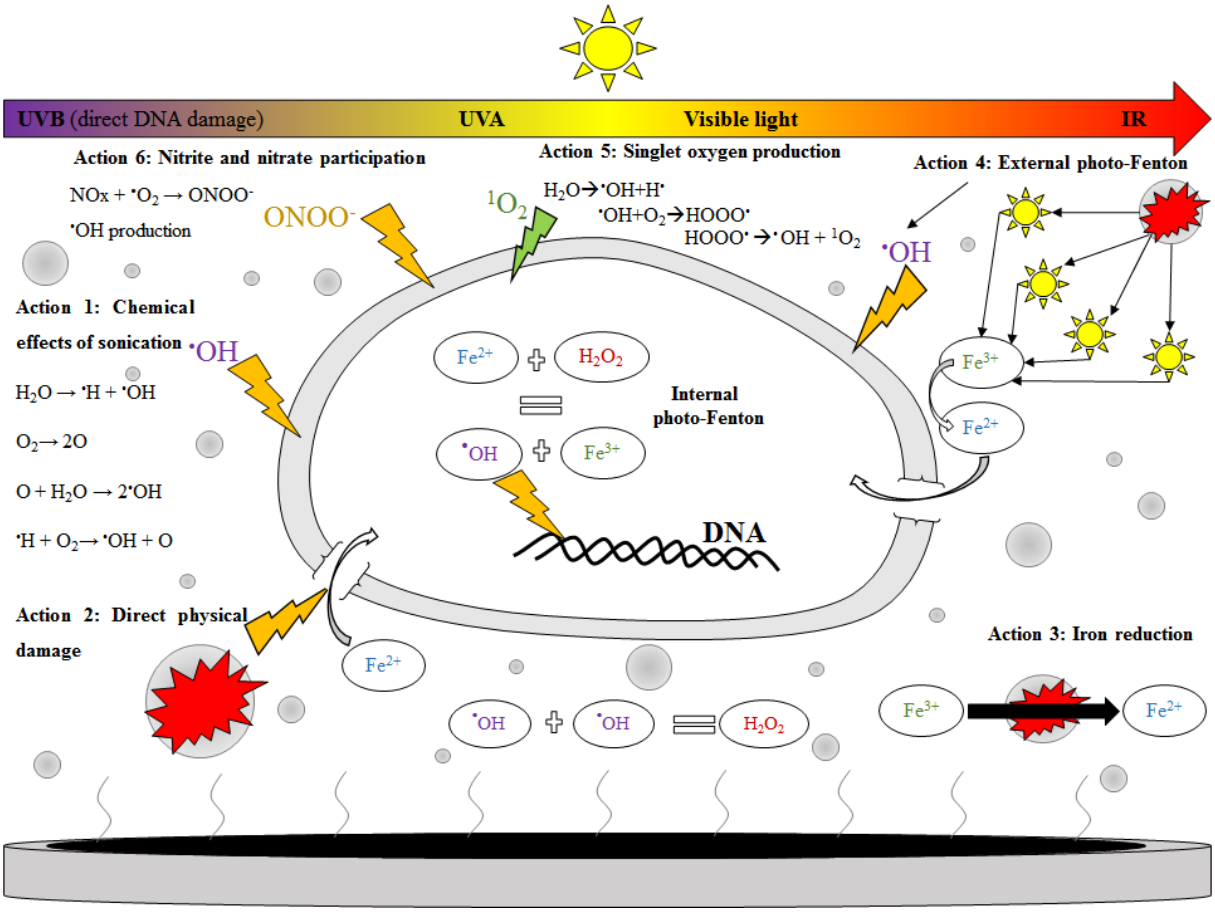


Figure 3 – Suggestion of the added actions sonication has towards bacterial inactivation, when coupled with photo-Fenton. The known photo-Fenton mechanisms suggested by literature are not displayed.

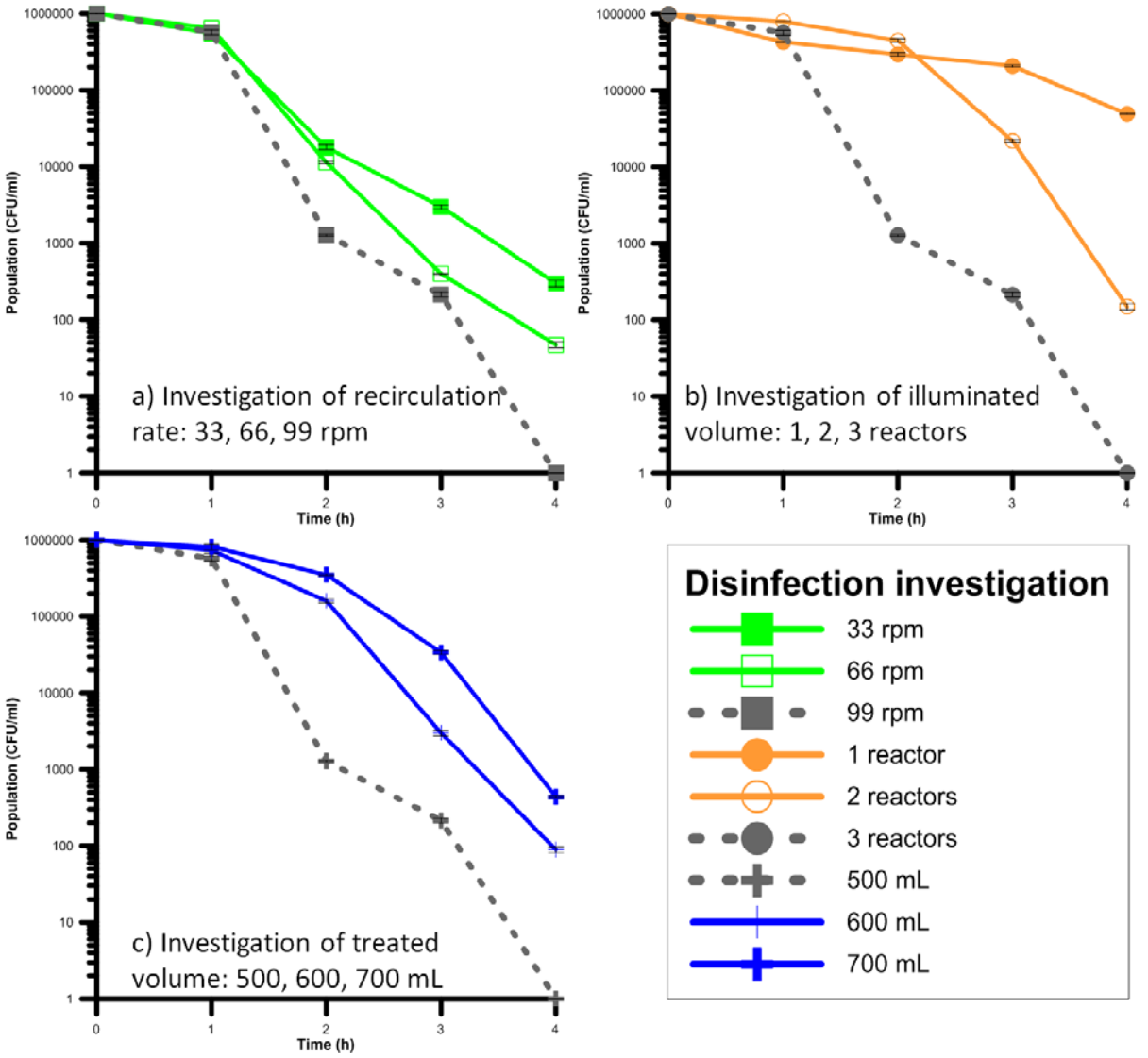


Figure 4 – Influence of the hydraulic characteristics of the experimental set-up, on the efficiency of the system. a) Investigation of the recirculation rate (33, 66 and 99 rpm), b) Investigation of the illuminated volume (1, 2 and 3 reactors) and c) Investigation on the effect of the treated volume (500, 600 and 700 mL).

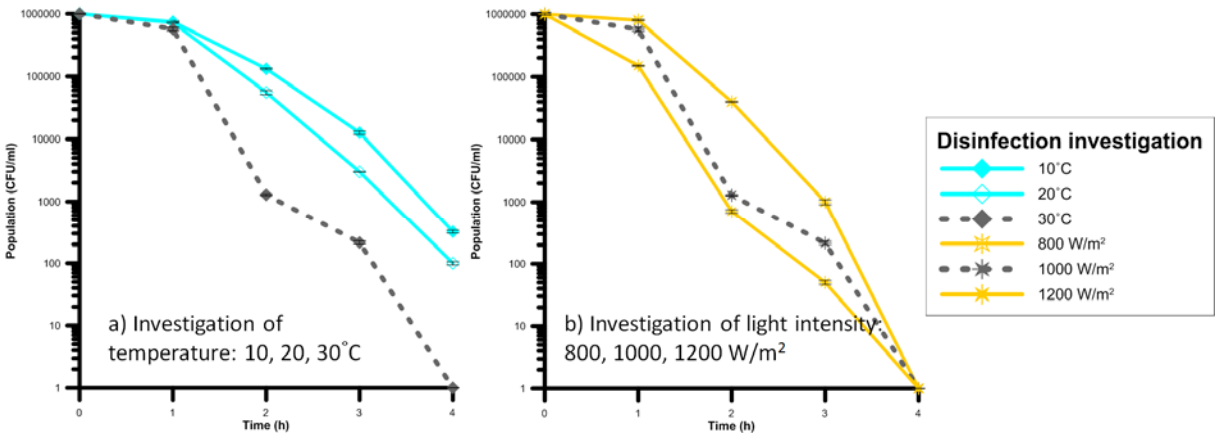
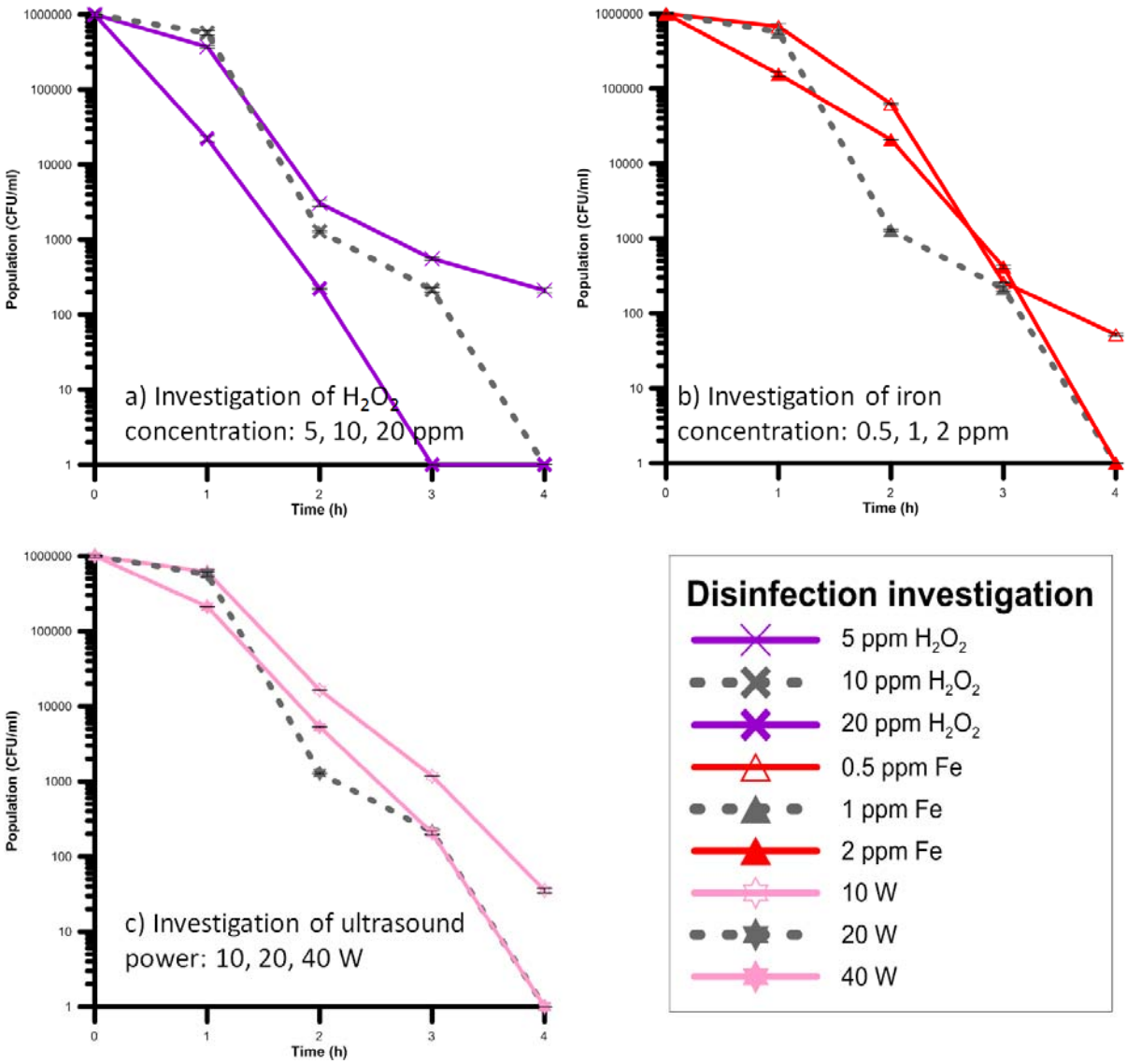


Figure 5 – Influence of the environmental parameters on the efficiency of the system. a) Investigation of temperature (10, 20 and 30˚C), b) Investigation of light intensity (800, 1000 and 1200 W/m^2).



736

737 Figure 6 - Influence of the Fenton reagents and the sonication intensity on the efficiency of the system. a)
738 Investigation of the H_2O_2 concentration (5, 10 and 20 ppm), b) Investigation of the iron concentration (0.5, 1,
739 and 2 ppm) and c) Investigation on the ultrasound power (10, 20 and 40 W).