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Inactivation of the waterborne pathogen *Cryptosporidium parvum* by photo-Fenton process under natural solar conditions

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ABSTRACT

Cryptosporidium is an important genus of emerging enteropathogens responsible for waterborne outbreaks worldwide. This parasite has a robust infective form (oocyst), which is highly resistant to the environmental conditions and to the conventional disinfection treatments of water. This work evaluates for the first time the photo-Fenton process against *Cryptosporidium parvum*. For that, a factorial design was used to study the combined effects of the Fe^{2+}/H_2O_2 concentration (5/10, 10/20 and 20/50 mg L^{-1}), pH (3, 5.5 and 8) and exposure time (2, 4 and 6 h) on the oocyst survival in distilled water under natural sunlight. The oocyst viability was determined by inclusion/exclusion of the fluorogenic vital dye propidium iodide. The variables Fe^{2+}/H_2O_2 concentration and exposure time showed statistically significant effects on the oocyst viability, as did the interaction of pH with Fe^{2+}/H_2O_2 concentration. The maximum oocyst inactivation rates corresponded to the combination of the highest concentration of Fe^{2+}/H_2O_2 (20/50 mg L^{-1}), the lowest pH value (3) and longest exposure times (4 and 6 h) (3.68 ± 1.38% and 6.39 ± 2.65%, respectively, *vs* 91.67 ± 3.63%, initial oocyst viability). Although further studies are needed to evaluate the influence of the water matrix and optimize the photo-Fenton process, the results obtained demonstrate the efficacy of this advanced oxidation process against *C. parvum* oocysts. The inactivation of this enteropathogen would probably ensure elimination of other less resistant infectious agents, providing an appropriate protection for the environment and, consequently, for human and animal health.

1. Introduction

Climate change, droughts and urban development occurred in recent years are factors that increased the pressures on water resources worldwide causing a scarcity and a deterioration of water [1]. This hydric stress can be reduced by enhancement and promotion of the reclamation of treated wastewater, decreasing also the discharges of sewage, and preventing the environmental pollution. The employ of the reclaimed water is an accepted practice worldwide [2], being reported in at least 60 countries [3]. Although, in May 2018, a proposal for a regulation of the European Parliament and of the Council on the minimum requirements for water reuse in agricultural irrigation was submitted, there is not yet guidelines, regulations or good management practices at the European Union level. However, several Member States, such as Cyprus, Greece, Italy, Malta, France, Portugal and Spain, have their own regulations regarding the permitted uses, as well as the parameters to be monitored [4,5].

For their use, reclaimed waters must comply different quality requirements that depend on their intended uses (urban, agricultural, aquifer recharge, landscape irrigation, environmental enhancement and others). The microbiological parameters are the most important for human and animal health due to the potential transmission of infectious diseases. All standards include a bacterial indicator to monitor the quality of reclaimed water (*Escherichia coli*, faecal coliforms or total coliforms); moreover, depending on the uses, other microbiological parameters are also contemplated (*Legionella*, *Salmonella* and helminth eggs). Furthermore, regulatory authorities in different countries adopted coliphages as viral indicators for monitoring of water reuse

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[6,7]. Regarding protozoan pathogens, *Cryptosporidium* has been selected as a reference organism for this group of parasites by several agencies [2,8,9], whereas other regulations have selected spore sulphate-reducing bacteria and *Clostridium perfringens* as an alternative protozoa indicator [4].

Cryptosporidium is an important genus of emerging enteropathogens that represents a significant cause of diarrhoeal disease worldwide. This parasite is transmitted by the faecal-oral route, directly from person to person or animal to person or indirectly via contaminated food and water, being the latter the most common route [10]. *Cryptosporidium* spp. have a robust infective form (oocyst), which is highly resistant to the environmental conditions and to the conventional disinfection treatments of water based on physical, chemical and biological methods [11]. Consequently, *Cryptosporidium* oocysts have been found in various different types of water worldwide (river water, drinking water and wastewater) [12–17] and this pathogen was the most common etiological agent identified in more than 381 waterborne outbreaks attributed to parasitic protozoa reported recently in developed countries [18].

Advanced oxidation processes (AOPs) are a group of related technologies that lead to the generation of reactive oxygen species, mainly $HO^{\cdot}$, which results in the oxidative degradation of pollutants and inactivation of several waterborne pathogens [19]. Photo-Fenton system is one of these processes, which received most attention in recent years [20]. The formation of $HO^{\cdot}$ in the homogeneous Fenton process in absence of a light source is produced when hydrogen peroxide (H_2O_2) is decomposed by Fe^{2+} ions dissolved in aqueous phase [21]:

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

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

$$Fe^{3+} + HO_2^{\bullet} \rightarrow Fe^{2+} + O_2H^+ \quad (3)$$

In presence of UV–vis irradiation of wavelengths below to 580 nm, a strong acceleration of reaction is observed, leading to much faster $HO^{\cdot}$ generation rates due to the light absorption of Fe^{3+} complexes, which are reduced to Fe^{2+} complexes, generating extra $HO^{\cdot}$ without the need for further addition of iron [21]:

$$Fe(OH)_2 + h\nu \rightarrow Fe^{2+} + 2HO^{\bullet} \quad (4)$$

The capacity of the homogeneous photo-Fenton process to remove bacterial, fungal and viral pathogens in water and wastewater have been demonstrated [22–25]. Nevertheless, to our best knowledge, photo-Fenton has not yet been evaluated against the protozoan parasite *Cryptosporidium*. The aim of this work was to study the effectiveness of this process in the inactivation of *Cryptosporidium parvum* oocysts in distilled water using several concentrations of Fe^{2+}/H_2O_2 and different pH values under natural solar radiation.

2. Experimental procedures

2.1. Cryptosporidium parvum oocysts

Cryptosporidium oocysts were collected from a naturally infected neonatal Friesian-Holstein calf. Concentration (0.04 M phosphate-buffered saline [PBS] pH 7.2 and diethyl ether), purification (discontinuous caesium chloride gradients), quantification (Neubauer haemacytometer) and molecular characterization were performed as previously reported [26]. Briefly, faeces were collected from a calf by rectal sampling and stored at 5 °C. Faecal material was then homogenised in 10–20 mL of PBS (0.04 M, pH 7.2), filtered through two sieves (mesh sizes 150 and 45 μm), shaken with diethyl ether (2:1, v/v) and concentrated by centrifugation at 2000 × *g* for 15 min at 4 °C. The resulting uppermost two layers were removed carefully and discarded, and the sediment was washed with PBS (0.04 M, pH 7.2) by centrifugation at 2000 × *g* for 15 min at 4 °C. *Cryptosporidium* oocysts were purified on discontinuous caesium chloride gradients of 1.05, 1.10 and 1.40 g mL^{-1} by centrifugation at 2000 × *g* for 30 min at 4 °C. Finally, the oocysts were counted in a modified Neubauer haemocytometer, with 0.16% malachite green solution as counterstain [27,28]. The isolate was identified as *C. parvum* by PCR amplification and sequence analysis of a ≈587-bp fragment of the small subunit rDNA gene (SSU-rDNA) [29].

2.2. Reagents and analytical measurements

Distilled water (pH 5.5, conductivity < 10 mS cm^{-1}, organic carbon < 0.5 mg L^{-1}, Cl^- 0.7–0.8 mg L^{-1}, NO_3^- 0.5 mg L^{-1}) was used as a reference solution for observing the inactivation kinetics under laboratory conditions, excluding the contribution or interference of any compound containing in other types of water. Distilled water was obtained by inverse osmosis from a distillation plant located at Plataforma Solar de Almería (PSA-CIEMAT).

Ferrous sulphate heptahydrate ($FeSO_4{\cdot}7H_2O$, Panreac, Barcelona, Spain) was used to obtain the desired concentrations of Fe^{2+} (5, 10 and 20 mg L^{-1} of Fe^{2+}). The concentrations of total iron, Fe^{2+} and Fe^{3+} were measured at the end of assays according to ISO 6332. Briefly, water samples were filtered with NY 0.20 μm CHROMAFIL® Xtra PET-20/25 syringe filters (Panreac) and mixed with 1 mL of 1.10-phenantroline (Sigma-Aldrich, Co., St. Louis, MO, USA) forming a coloured complex between Fe^{2+} and 1,10-phenanthroline. For determination of total iron, ascorbic acid was added to reduce Fe^{3+} to Fe^{2+}. Then, the absorbance was measured with a spectrophotometer T-60U (PG Instruments Ltd., Lutterworth, United Kingdom) at 510 nm using glass cuvettes (1 cm path length). The concentrations of Fe^{2+} and total iron were determined employing the corresponding calibration curve between 0.25 and 10 mg L^{-1}, being the samples with a concentration above 10 mg L^{-1} diluted accordingly. The concentration of Fe^{3+} was calculated by the difference between total iron and Fe^{2+}.

In this work, the concentration ratio of Fe^{2+} to H_2O_2 used was approximately 1:2 with concentrations of 10, 20 and 50 mg L^{-1} by addition of H_2O_2 directly in the reactor from a stock solution (8000 mg L^{-1}, Merk, Darmstadt, Germany). The H_2O_2 concentration was measured at 410 nm with a spectrophotometer T-60U (PG Instruments Ltd.) in glass cuvettes (1 cm path length) at the end of the assays following DIN 38409 H15 [22], based on the formation of a yellow complex from the reaction of H_2O_2 with titanium (IV) oxysulfate (Honeywell Riedel de Haën AG, Seelze, Germany). H_2O_2 concentration was determined using a standard curve linear between concentration ranges of 0.5–60 mg L^{-1}.

Different pH values were evaluated in this study (3, 5.5 and 8). For that, pH of the samples was adjusted using solutions of sulphuric acid 1 M (H_2SO_4, Panreac) and sodium hydroxide 1 M (NaOH, Acros Organics, Geel, Belgium) before and after addition of $FeSO_4{\cdot}7H_2O$ and H_2O_2, being also measured at the end of each exposure time (2, 4 and 6 h) with a portable pH-meter LAQUAact D-71 (HORIBA, Ltd., Northampton, United Kingdom).

2.3. Solar radiation

Natural solar experiments were performed at the PSA-CIEMAT, located in the Tabernas Desert (Almería, Southern Spain: latitude, 37°84'N; longitude, 2°34'W; altitude, 500 m) in July 2017. Incoming UV radiation (direct plus diffuse radiation from all directions) was measured between 295 nm and 385 nm (part of UV-A and UV-B) with a global UV radiometer placed horizontally (model CUV5, Kipp & Zonen, Delft, The Netherlands). The radiometer provides data in terms of incident irradiation (W m^{-2}), which is defined as the energy rate of solar radiant incident on a surface per unit of area. The parameter Q_{UV} is the accumulative energy per unit of volume (kJ L^{-1}) received in the reactor and is calculated according to Eq. (5):

Table 1
Experimental domains and coding of the three variables investigated.

Coded value	Real values		
	Fe^{2+}/H_2O_2 (mg L^{-1})	pH	Exposure time (h)
−1	5/10	3	2
0	10/20	5.5	4
1	20/50	8	6

$$Q_{UV,n} = Q_{UV,n-1} + \frac{\Delta t_n \cdot \overline{UV}_{G,n} \cdot A_r}{V_t}; \quad \Delta t_n = t_n - t_{n-1} \tag{5}$$

where $Q_{UV,n}$ and $Q_{UV,n-1}$ represent the UV energy accumulated per litre (J L^{-1}) at times t_n and t_{n-1}, respectively; Δt_n is the exposure time of the sample (s); $\overline{UV}_{G,n}$ is the average incident UV radiation on the irradiated area (W m^{-2}); A_r is the illuminated area of reactor (m^2); and V_t is the total volume of sample (L).

2.4. Experimental design

A 3 × 3 first-order full factorial design was used to study the combined influence of several Fe^{2+}/H_2O_2 concentrations (5/10, 10/20 and 20/50 mg L^{-1}), three pH values (3, 5.5 and 8) and different exposure times (2, 4 and 6 h) in the inactivation of *C. parvum* oocysts by photo-Fenton process [30]. Statistically significant effects on oocyst viability were identified by analysis of variance (ANOVA). The effects of those factors shown to be significant ($P < 0.05$) by ANOVA were modelled by means of least-square multiple regression. Analyses were performed with Statgraphics Centurion v.16.1 (©1982-2011 Statgraphics Technologies, Inc., The Plains, Virginia, USA). Table 1 summarizes the different combinations of these variables according to the experimental design.

Borosilicate-glass 3.3 low form beakers of 35 mm of diameter were filled with 30 mL of distilled water containing the three Fe^{2+}/H_2O_2 concentrations at the three pH values stablished. Then, the samples were spiked with 6×10^6 purified *C. parvum* oocysts and exposed to natural solar radiation during a maximum exposure time of 6 h, so that the mid-point of each exposure time occurred at noon on each day (from 11:00 to 17:00 h, local time). During the experiments, samples were magnetic stirred at 250 rpm on a horizontal platform (IKA® big squid white, IKA®-Werke GmbH & Co. KG, Staufen, Germany) and temperature was monitored every hour using a thermometer Checktemp HI 98509-1 (Hanna Instruments®, Eibar, Spain).

At 2, 4 and 6 h of exposure time, total volume of the samples was removed and centrifuged at 2000 × *g* for 15 min. The supernatants were discarded and the sediments thus obtained were resuspended in 2 mL of PBS (0.04 M, pH 7.2) and used to evaluate the oocyst viability. All tests were performed in duplicate.

2.5. Viability assays

The viability of *C. parvum* oocysts was determined by inclusion/exclusion of the fluorogenic vital dye propidium iodide (PI) and a further modification that includes an immunofluorescence antibody test to verify oocyst identification [31,32]. Briefly, 200 µL of the sediments were incubated with 15 µL of PI (Sigma-Aldrich, Co.) working solution [1 mg mL^{-1} in PBS (0.1 M, pH 7.2)] and 15 µL of monoclonal antibodies labelled with fluorescein isothiocyanate (FITC) (AquaGlo™ G/C Direct Test, Waterborne Inc., New Orleans, LA, USA) at 37 °C, for 30 min [26]. Then, the samples were washed three times in PBS (0.04 M, pH 7.2) at 10,000 × *g*, for 5 min at room temperature. Oocysts were identified first under a FITC filter (excitation at 450–480 nm; barrier at 515 nm) before being examined for PI inclusion/exclusion under a PI filter (excitation at 510–550 nm; barrier at 590 nm). The proportions of ruptured (ghost), PI-positive (dead), and PI-negative (viable) oocysts were quantified in an epifluorescence microscope equipped with a Nomarski differential interference contrast, FITC and PI filters (Eclipse 50i, Nikon Corporation, Tokyo, Japan). The results are shown as the percentage of PI-negative (viable) oocysts [Eq. (6)], which were determined for each assay after triplicate counts of more than 100 oocysts.

$$Oocyst \quad viability\ (\%) = \left(\frac{PI - negative\ oocysts}{Total\ oocysts}\right) \times 100 \tag{6}$$

3. Results and discussion

This is the first study that evaluates the photo-Fenton process against the waterborne protozoan parasite *Cryptosporidium*. For that, a factorial design was applied to study the combined effects of the Fe^{2+}/H_2O_2 concentration, pH and exposure time on the survival of *C. parvum* oocysts in distilled water under natural conditions of solar radiation. The use of factorial designs offers important advantages, in comparison with one-factor-at-a-time experiments, since they allow effects to be investigated with many fewer experiments without changing reliability in the obtained results, the detection of factor interactions and the identification of the response-variable maxima, in addition to facilitate system-modelling [33]. This type of experimental design is most often used for manipulative experiments in order to evaluate the influence of several factors in the survival of different organisms, including *Cryptosporidium* [30,33–36]. Consequently, a 3 × 3 first-order full factorial design was selected to study the combined influence of three factors [pH (*P*), Fe^{2+}/H_2O_2 concentration (*C*) and exposure time (*T*)], at three levels of each factor, in the oocyst viability.

The isolate of *C. parvum* used in the present study showed an oocyst viability of 91.67 ± 3.63%. The weather conditions during the experiments were sunny days and the maximum local noon UV irradiance recorded within this exposure period was 46.08 ± 0.56 W m^{-2}, reaching a Q_{UV} values of 9.20 ± 0.92, 19.12 ± 0.99 and 28.31 ± 0.04 kJ L^{-1} after 2, 4 and 6 h of exposure to natural sunlight (Fig. 1).

The results of ANOVA of the data obtained for the inclusion/exclusion of PI are summarized in Table 2. The minimum values of oocyst viability corresponded to the combination of the highest concentration of Fe^{2+}/H_2O_2 (20/50 mg L^{-1}), the lowest pH value (3) and longest exposure times (4 and 6 h) (rows 6 and 13, 3.68 ± 1.38% and 6.39 ± 2.65%, respectively, *vs* 91.67 ± 3.63%, initial oocyst viability). Moreover, low oocyst viability was determined at pH 5.5 using a Fe^{2+}/H_2O_2 concentration of 20/50 mg L^{-1} after an exposure time of

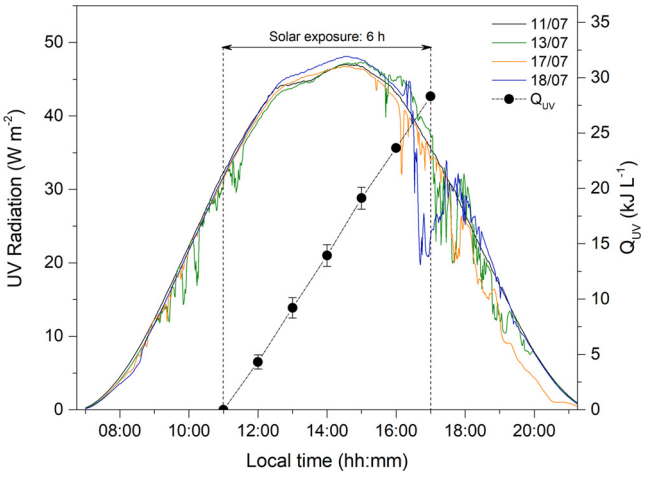

Fig. 1. UV irradiance (295–385 nm) measurements and average of Q_{UV} calculated during the assays of the homogeneous photo-Fenton process against *C. parvum* carried out under natural solar radiation in PSA-CIEMAT, Spain.

Table 2
Design of the experiments reported in the present study, showing response (oocyst viability, %) obtained in each run and results of analysis of variance to investigate the effects of Fe^{2+}/H_2O_2 concentration (*C*), pH (*P*) and exposure time (*T*) on oocyst viability (%). The *C*, *P* and *T* values shown are coded values (see Table 1).

Row	*C*	*P*	*T*	Observed value	Fitted value	Studentized residual
1	−1	−1	1	54.29	52.66	0.1575
2	−1	−1	−1	75.41	88.40	−1.5107
3	0	−1	1	50.80	30.40	1.8813
4	0	1	1	33.17	34.14	−0.0759
5	−1	1	1	31.12	34.91	−0.3696
6	1	−1	0	3.68	26.01	−2.2581
7	1	−1	−1	66.65	43.88	2.6459
8	1	0	−1	47.79	56.49	−0.6967
9	0	0	0	54.80	50.14	0.3362
10	1	1	1	37.35	33.37	0.3399
11	1	1	−1	72.99	69.11	0.3407
12	1	1	0	52.75	51.24	0.1222
13	1	−1	1	6.39	8.14	−0.1490
14	1	0	1	9.35	20.76	−0.9107
15	−1	1	−1	73.75	70.64	0.3248

Source	Coefficient	Sum squares	df	Mean squares	F ratio	P value
Constant	50.139					
C	−11.515	1446.39	1	1446.39	7.64	0.0200
P	1.868	40.48	1	40.48	0.21	0.6537NS
T	−17.867	3737.10	1	3737.10	19.74	0.0012
PC	10.746	1116.35	1	1116.35	5.90	0.0356

NS, not significant.
df, degrees of freedom.
Durbin-Watson statistic = 2.9896 (*P* = 0.9335).

6 h (row 14, 9.35 ± 2.26%). Nevertheless, only Fe^{2+}/H_2O_2 concentration and exposure time showed statistically significant effects on the oocyst viability, as did the interaction of pH with Fe^{2+}/H_2O_2 concentration ($P < 0.05$). These variables were analysed using response surface methodology. Thus, multiple regression analysis resulted in the following equation Eq. (7):

$$Oocyst\ viability\ (\%) = 50.139 - 11.515C - 17.867T + 10.746PC + 1.868P \quad (7)$$

where *C*, is the concentration of Fe^{2+}/H_2O_2; *T*, is the exposure time; *PC*, is the interaction between the pH and the concentration of Fe^{2+}/H_2O_2; R^2 = 76.76%; R^2 adjusted for df = 67.46%; standard error of estimate = 13.76; mean absolute error = 8.26. This statistic model explains to 76.76% of oocyst viability; *P* value of the model was 0.0033, which indicates the adjustment of the model to the observed data is statistically significant ($P < 0.05$). The response surface plots of the oocyst viability defined by this equation when one of the three variables is fixed at its domain-central value (i.e. code value zero) are shown in Fig. 2. According to the Durbin-Watson test, which detects autocorrelation in the residuals from a regression analysis (to determine the existence of some kind of dependence on the order in which values were entered on data matrix), a statistic value of 2.986 was obtained ($P = 0.9335$). Autocorrelation means that the errors of adjacent observations are correlated. If the errors are correlated, then least-squares regression can underestimate the standard error of the coefficients. Underestimate standard errors can make predictors seem significant, when in fact they are not. In the present study, since the *P* value is greater than 5% there is not indication of serial autocorrelation of the residuals at significance of 5%.

The individual effects of the three parameters evaluated (*P*, *C* and *T*) on the viability of *C. parvum* oocysts in distilled water are showed in Fig. 3. The largest coefficient with a negative sign corresponded to the exposure time. Although, it is well known that solar exposure by itself produces the inactivation of different microorganisms, the exposure time is not often used in experiments carried out under natural sunlight,

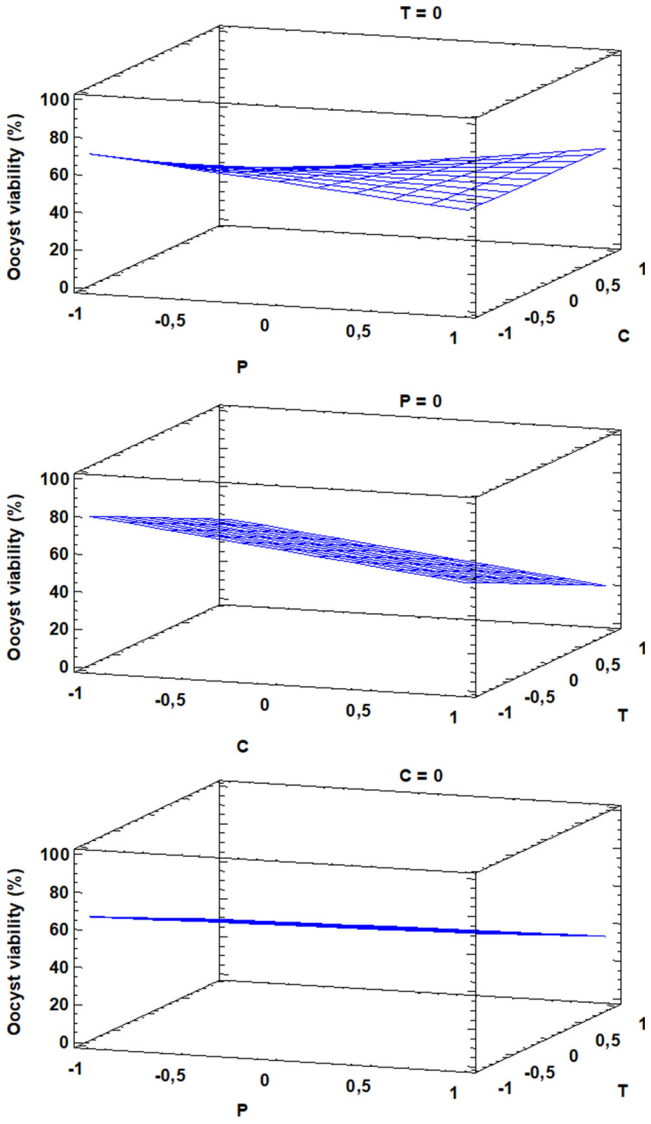

Fig. 2. Response surface plots of *C. parvum* oocyst viability (%) as a function of Fe^{2+}/H_2O_2 concentration (*C*), pH (*P*) and exposure time (*T*). In each plot one of the three variables is fixed at its domain-central value (i.e. coded value zero).

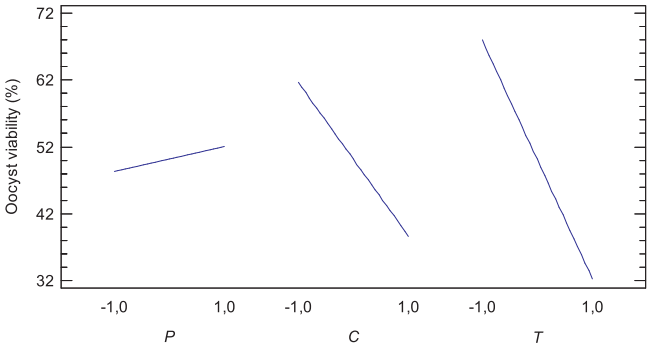

Fig. 3. Graphical representation of the individual effects of pH (*P*), concentration of Fe^{+2}/H_2O_2 (*C*) and exposure time (*T*) on the viability of *C. parvum* oocysts in distilled water under natural solar radiation.

due to the solar irradiance vary constantly [37]. In the present study, the UV irradiances (295–385 nm) oscillated between a minimum of 29.65 ± 1.62 W m^{-2} and a maximum of 46.08 ± 0.56 W m^{-2}. At high intensities of irradiation, the exposure time is an important

variable, but at low levels of irradiation intensity this parameter has a null effect on the viability of *C. parvum* oocysts [36].

Furthermore, the concentration (*C*) and the interaction between pH and concentration of Fe^{2+}/H_2O_2 (*PC*) had statistically significant effects on oocyst viability, but with contrary sign. The pH of the solution is a very important parameter in photo-Fenton processes due to this variable has a strong influence in the speciation and solubility of iron in water, being the optimum pH ≤ 3. At this pH value, the dominant species of iron is Fe^{2+}, but as pH increases, the species of iron in the solution change. At pH 5–8, the principal species are Fe^{2+} and $Fe(OH)^0_{2(aq)}$, whereas at pH ≥ 8, the dominant species are $Fe(H_2O)_4(OH)_2$ and $Fe(OH)^0_{2(aq)}$ [21,37]. In the present study, three pH values were evaluated: 3, 5.5 and 8, which were maintained stable throughout the experiments, except in the samples of pH 8 ($P < 0.0001$), i.e., 3.23 ± 0.06 and 3.14 ± 0.10; 5.46 ± 0.14 and 5.36 ± 0.23; 8.11 ± 0.15 and 7.31 ± 0.08, initial and final pH values, respectively. At pH 3, the concentration of dissolved iron diminished significantly and independent of the initial concentration, not observing statistically significant differences among the three iron concentrations stablished (final Fe^{2+} concentration of 1.29 ± 0.34, 1.20 ± 0.68 and 1.17 ± 0.28 mg L^{-1} of Fe^{2+} for initial concentration of 5, 10 and 20 mg L^{-1}, respectively). This can be due to the quick precipitation of iron in forms of aqua complexes after the addition of H_2O_2 [21]. In samples with pH values of 5.5 and 8, the concentrations of dissolved iron (Fe^{2+}, Fe^{3+} and total iron) were lower than the detection limit (0.25 mg L^{-1}). Both at pH 5.5 as at pH 8, iron species can precipitate and generate complexes in the solution, which cause changes in the coloration of the sample and increase the number of suspended particles in the water that absorb and scatter the light, affecting negatively to the penetration of sunlight and therefore reducing its efficacy in the inactivation of microorganisms [38]. Consequently, the strong influence of the pH in the solubility/precipitation of iron salts makes that the interaction pH/concentration of Fe^{2+}/H_2O_2 (*PC*) has a high and significant relevance in the proposed model. Thus, at the highest pH (8, coded value = 1) and concentration values (20 mg L^{-1}, coded value = 1), the *PC* coefficient has a positive sign and hence it shows a protective effect in the oocyst viability. However, at the lowest pH (3, coded value = −1) and the highest concentration (20 mg L^{-1}, coded value = 1), the *PC* coefficient has a negative sign and the oocyst inactivation is increased. At the highest pH values (8, coded value = 1) and the lowest concentration of Fe^{2+}/H_2O_2 (5 mg L^{-1}, coded value = −1), the *PC* coefficient has also a negative sign, but its effect is balanced by the *C* coefficient, with a positive sign in this case, without apparently affecting the oocyst survival.

On the other hand, as it was demonstrated in a previous study carried out under simulated solar radiation [39], the variable pH alone did not have influence in the inactivation of *C. parvum* oocysts in distilled water. Thus, at the lowest Fe^{2+}/H_2O_2 concentration (5/10 mg mL^{-1}) and after a exposure time 2 h, the oocyst viability was 75.41 ± 4.73% and 73.75 ± 3.09% at pH values of 3 and 8 (row 2 and 15), respectively. Nevertheless, it is well known that pH affects to the zeta potential of the particles in suspension. In this study, the observation of the samples under bright field microscopy revealed aggregates of iron particles on the surface of the oocysts, which number increases as pH value and iron concentration are higher. Aggregation of iron particles on oocysts is a consequence of the different surface charges: at neutral-alkaline pH, the oocyst wall has a strong negative superficial charge [40], whereas iron species are positively charged [21]. This fact could favour the photocatalytic disinfection as the short-lived $HO^{\cdot}$ are generated directly on the surface of the oocyst wall.

Photo-Fenton process has been widely evaluated against virus, bacteria, fungi, and the nematode parasite *Ascaris suum* using several iron compounds and concentrations, different ratios of Fe to H_2O_2 (which vary from 1 to 5–10 wt/wt of Fe^{2+} to H_2O_2), several types of water (distilled water, MilliQ water, simulated and real municipal wastewater effluents) under simulated and natural solar conditions [41]. Thus, a reduction of 80% in the infectivity capacity of Hepatitis A virus was reported after 6 h of exposure to natural sunlight in distilled water spiked with 10^5 plaque-forming units (PFU) mL^{-1} at a Fe^{2+}/H_2O_2 concentration of 10/20 mg L^{-1} and pH ≈ 4 [24]. Also, under natural solar radiation, Ortega-Gómez et al. [42] observed a complete inactivation of *E. coli* and total coliforms using a secondary effluent from a municipal wastewater treatment plant containing a Fe^{2+}/H_2O_2 concentration of 20/50 mg L^{-1} at neutral pH after 60 min. In addition, a complete inactivation of *Fusarium solani* was determined after 4 h of exposure to real sunlight using a simulated municipal wastewater treatment plant effluent and a Fe^{2+}/H_2O_2 concentration of 5/10 mg L^{-1} at pH 3 [22]. Similarly, the inactivation of *Phytophthora capsici* zoospores was achieved in distilled water using the same concentration of Fe^{2+}/H_2O_2, at pH < 4 after 2 h of exposure time under natural conditions [43]. In the present study, the maximum oocyst inactivation rate was observed when the assays were carried out using a Fe^{2+}/H_2O_2 concentration of 20/50 mg L^{-1}, at pH 3, after 4–6 h of exposure to natural sunlight. The oocyst viability was determined using inclusion/exclusion of the fluorogenic vital dye PI, an indicator of the integrity of the oocyst wall [31]. This staining method is quick, simple and relatively inexpensive and provides useful information in studies on the influence of environmental factors on the survival of *Cryptosporidium* spp. oocysts, but this technique overestimates the oocyst infectivity in comparison with cell culture methods and bioassays with murine models [44,45]. For this reason, the oocyst viability values obtained are conservative and correspond to lower levels of infectivity, as previously demonstrated [36].

In the present study, a Fe^{2+}/H_2O_2 concentration ratio of approximately 1:2 was used, since this ratio showed the best results according with different authors [37]. Thus, H_2O_2 concentrations of 10, 20 and 50 mg L^{-1} were employed, which do not affect to the survival of *C. parvum* as it was demonstrated in previous studies carried out under natural sunlight [46]. Moreover, H_2O_2 was completely consumed after 2 h of exposure to natural solar radiation, independently of the pH and the initial concentration. This quick reduction in the H_2O_2 concentration could be due to the reaction with Fe^{2+} and to the temperature reached in the water since, at temperatures higher than 30 °C, H_2O_2 decomposes to H_2O and O_2 [37]. In all of the assays carried out in the present study, water temperatures > 37 °C were registered after 1 h of exposure to natural sunlight.

Temperature does not only affect the reaction kinetics but also it is one of the most important abiotic factors that affect the survival of *C. parvum* in the environment [47,48]. Temperature values of 30–50 °C cause a decrease in the viability of *Cryptosporidium* due to the phenomenon of melting point of the fatty acids and hydrocarbons present in the oocyst wall and an increase of the metabolism activity of the amylopectin [48–51]. Furthermore, high temperatures can induce the spontaneous excystation of *C. parvum* oocysts [52], in which a small percentage of the sporozoites may excyst when the oocysts are incubated at 37 °C in the absence of any other stimulus, which makes their survival impossible because they are in a different environment provided by the host [53]. Moreover, a strong synergistic effect between the optical and thermal effects of the solar radiation was described at temperatures above at 45 °C [54,55]. Throughout the assays, temperature ranged between 24.6 ± 1.73 °C and 44.4 ± 4.10 °C, reaching a maximum value of 47.3 °C after 5 h of exposure to sunlight (16:00 h local time). Therefore, in addition to the photo-Fenton process, the reductions observed in the oocyst viability were also consequence of the higher temperature values reached inside the samples as previously demonstrated by Gómez-Couso et al. [56]. As mentioned previously, the intensity of UV radiation ranged between a minimum of 28.5 W m^{-2} and a maximum of 48.1 W m^{-2}, Factorial design does not include the parameters temperature and intensity of solar radiation, which vary throughout of the experiments carried out under field conditions. These variations can cause imbalances in the proposed model and, for this reason, the values provided by the model do not fit

completely with the observed data in the oocyst viability.

Finally, the efficiency of the photo-Fenton process depends on different factors like the water matrix, the pH, the intensity of the radiation and the target microorganism. The chemical composition of the water is an important factor due to organic matter is a natural competitor of the hydroxyl radicals, reducing the efficacy of process [41]. Moreover, it was proved that the use of the chelates at neutral pH enhance the efficacy of photo-Fenton process because these compounds help to maintain the iron dissolved in the water [37]. Although further studies are needed to evaluate the influence of the water matrix on the efficacy of the photo-Fenton process against *C. parvum* oocysts, the results obtained in the present study confirm the high resistance of this waterborne protozoan parasite to disinfection treatments in comparison with other microorganisms as it was reported previously [11]. The inactivation of this enteropathogen would probably ensure elimination of other less resistant infectious agents, providing an appropriate protection for the environment and, consequently, for human and animal health.

4. Conclusion

The results obtained in the present study demonstrate the efficacy of the photo-Fenton process to inactivate the emerging protozoan parasite *Cryptosporidium* in distilled water under natural sunlight. However, high Fe^{2+}/H_2O_2 concentrations (20/50 mg L^{-1}), low pH values (≤ 5.5) and long exposure times (4 and 6 h) are required to obtain good inactivation rates of *C. parvum* oocysts in distilled water. Therefore, although further optimization is needed, homogeneous photo-Fenton process represents a sustainable and promising alternative to the disinfection methods currently used in water reclamation.

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