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Resistance and induction of viable but non culturable states (VBNC) during inactivation of *E. coli* and *Klebsiella pneumoniae* by addition of H_2O_2 to natural well water under simulated solar irradiation

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ABSTRACT

Inactivation of *E. coli* and *Klebsiella pneumoniae* by addition of H_2O_2 10 mg L^{-1} into natural well water samples containing natural total iron concentrations (around 0.3 mg L^{-1}) under simulated solar light was followed by bacterial culturability (plate count) and viability (DVC-FISH). Results showed that culturability of both bacteria was totally reduced while viability was only completely depleted for *E. coli* in well water samples depending of total iron concentration. Post-irradiation effects in presence of residual H_2O_2 showed that viability of both bacteria kept dropping being totally reduced for *E. coli* cells while *K. pneumoniae* decreased only 1-log. SEM micrographs showed that *E. coli* and *K. pneumoniae* cells underwent morphological changes and size reduction according to VBNC states. Different dark and photo-induced processes where physical-chemical features of groundwater samples play an important role could be responsible of bacteria abatement.

1. Introduction

In Latin America, around 31% of rural communities use groundwater as drinking water source ((PNUMA), 2010). However, drawbacks linked to low index of water sanitation and hygiene (WASH) and agricultural activities in these communities threat groundwater sources causing their microbiological and chemical pollution (WWAP (Programa Mundial de Evaluación de los Recursos Hídricos de la UNESCO)., 2019).

Low-cost processes are required to produce chemical and microbiological safe drinking water from groundwater. In this context, solar disinfection (SODIS) has emerged as a promising alternative in developing countries to produce drinking water in isolated rural communities (McGuigan et al., 2012). However, SODIS exhibits some drawbacks related to treated water volume and possible bacteria regrowth in further dark storage periods. A promising process involving photo-Fenton reactions to inactive bacteria in water has been widely explored in the literature (Giannakis et al., 2016; Ortega-Gómez et al., 2014), however, in 2010, Sciacca et al., (Sciacca et al., 2010) claimed that the simple adding of H_2O_2 to natural Sahelian surface waters could enhance SODIS process avoiding further bacteria culturability regrowth. These authors suggested that natural iron (dissolved or colloidal) present in surface waters could participate alongside with H_2O_2 in Fenton, photocatalytic and photo-Fenton reactions leading to the photoinduction of hydroxyl radicals ($^{\bullet}OH$) responsible of bacteria culturability abatement. This approach was also explored by Ndounla et al.,(Ndounla et al., 2014) in Burkina Faso well waters where *E. coli* and *Salmonella* spp. culturability was completely reduced without further dark regrowth in sunlight-irradiated CPC reactors. Recently, some of us argued that adding of H_2O_2 (10 mg L^{-1}) to well waters from Colombia (South America) which already contain natural total iron amounts around 0.3 mg L^{-1} led to the bacteria inactivation after 6 h of simulated sunlight irradiation (H.M. Gutiérrez-

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Zapata et al., 2017a). This time, viability (by DVC-FISH) instead culturability was followed and apparently, groundwater chemical matrix may exert an important role in bacteria abatement since bicarbonates and fluoride natural concentrations exhibited positive effects. Culturability involves the capacity of bacterial cells to grow and form colonies on conventional culture media whereas viability implies bacterial cells exhibiting metabolic and physiologic activity (Stokell and Steck, 2012).

Precisely, physical-chemical features of natural well water samples make very interesting these water sources in sunlight photo-induced chemical reactions. It is well known that presence of NO_3^-, NO_2^-, carbonates/bicarbonates, iron, H_2O_2, and chromophoric dissolved organic matter (CDOM) in natural surface waters can induce the generation of reactive oxygen species (ROS) such as hydroxyl radicals ($^{\bullet}OH$) carbonyl radical ($CO_3^{-\bullet}$) and singlet oxygen ($^1O_2$) under sunlight irradiation (Vione and Scozzaro, 2019). Groundwater contains all these chemical species excepting CDOM since due to natural chemical and biological events, organic matter in these waters may be frequently found as formiates and acetates. Furthermore, groundwater exhibits the presence of iron (hydr)oxides such as goethite (α-FeOOH) and lepidocrocite (γ-FeOOH) along with inosilicate minerals named piroxenes and amphiboles composed of Si, Ca, Mg, among other cations, which can incorporate adsorbed ferrous ions coming from anaerobic redox processes (Appelo and Postma, 2005).

In 2018, some of us reported the building and evaluation of an integrated system based in helio-photochemical H_2O_2-processes coupled with conventional drinking water systems such as fast sand filtration and chlorination obtaining encouraging results to simultaneously inactivate bacteria and remove organic pollutants from 30 L of groundwater samples. However, it was found that *Klebsiella pneumoniae* strain was more resistant than *E. coli* to the treatment requiring high sunlight doses (Alvear-Daza et al., 2018a).

Although the promising results about the use of simple H_2O_2 addition and sunlight irradiation to natural surface or well waters show that it is possible to induce bacteria inactivation by enhancement of photo-induced abiotic events responsible of ROS generation, some issues remain yet to explore. For instance, (i) what happens if bacteria viability and culturability are simultaneously followed? This is a key point since it is well known that bacteria, under oxidative stress, can enter into viable non culturable states (VBNC). This is a strategy adopted by microorganism to survive under unfavorable conditions (such as oxidative stress) where cells are unable to form colonies in solid media leading possibly to wrong results if culturability is exclusively followed (Kim et al., 2018). Bacteria cells in VBNC states have been found after chlorination and other disinfecting processes and important bacteria monitoring discrepancies between conventional plate-count and viability techniques have been reported (Chen et al., 2018; Kong et al., 2016). (ii) Could any kind of bacteria strains be effectively inactivated by this photo-chemical treatment? Giannakis et al.,(Giannakis et al., 2018) and Serna-Galvis et al., (Serna-Galvis et al., 2019) have recently reported that even antibiotic-resistant bacteria strains such as *Klebsiella pneumoniae, Staphylococcus aureus* and *E. coli* can be inactivated by solar photo-Fenton processes monitored by cell culturability. However, as it was described before, bacteria cells and especially those resistant to oxidative stress can induce the formation of cells in VBNC states. For instance, *K. pneumoniae* is a bacteria cell exhibiting not only antibiotic resistance but also extracellular catalases, strong ability to iron capture, and the presence of a polysaccharide capsule offering protection to the cell against oxidative stress (Goldberg and Hochman, 1989; Paczosa and Mecsas, 2016). (iii) Further bacteria inactivation in dark storage periods is also a critical issue. Some authors have demonstrated by following culturability that these dark processes can also induce bacteria inactivation after the photochemical treatment (Rincón and Pulgarin, 2007). However, to our knowledge there are not reported studies about the following of this phenomenon by viability techniques.

Herein, it is reported a detailed study about the inactivation of two bacteria strains *E. coli* and *Klebsiella pneumoniae* by photochemical processes induced by addition of H_2O_2 to natural well waters containing natural iron concentrations. Bacteria inactivation was followed by culturability (plate count-PC) and viability techniques (DVC-FISH). Moreover, post-irradiation events were also explored after the photochemical treatment in presence and absence of residual H_2O_2, specifically in samples where total bacteria inactivation was not achieved. Morphological changes in cells by SEM micrographs were evaluated during the photo-chemical inactivation. Effect of colloidal matter was also evaluated on bacteria inactivation and residual H_2O_2 was measured.

2. Experimental section

2.1. Well water samples

Natural well water was taken by twice (the second one W-W2 some months later) by using standard procedures from a unconfined aquifer (depth 6 m, i.d. 30 cm) located in a small rural community (3°21′03.38″ N 76°26′24.51″ S) in Colombia by using a submersible pump with a flow rate of 100 mL min-1 (further details in supplemental material). The pump was disinfected with sodium hypochlorite solution and then samples were stored at 4C. Further physical-chemical characterization was evaluated by standard procedures (more information is provided in supplemental material).

2.2. Reagents

All reagents were used without further purification (details are provided in supplemental material).

2.3. Analysis of solids recovered from well-water samples

Solids obtained from well water samples (by filtration) were evaluated by SEM-EDS (further details provided in supplemental material).

2.4. Microbiological analysis of bacteria strains

Culturability and viability of *E. coli* K-12 *and Klebsiella pneumoniae* laboratory strains (ATCC 23716 and ATCCBAA-1705 respectively) were followed by a direct viable counting coupled with fluorescent in situ hybridization (DVC-FISH) and classic plate-count (PC) procedures previously described by us (Alvear-Daza et al., 2018b; Gutiérrez-Zapata et al., 2017c). Each sample was measured by triplicate (further details provided in supplemental material).

2.5. Dark and simulated sunlight irradiated experiments

A solar simulator (Hanau Suntest AM-1 300 W) equipped with a Xenon lamp was used to perform photochemical experiments and its radiant flux was monitored. Several Pyrex bottles of 100 mL were filled with 80 mL of well water samples (W-W or W-W2) and 10 mg L^{-1} H_2O_2 were added. Then, *E. coli* and *K. pneumoniae* were spiked into the well-water samples until reaching a suitable concentration (10^7 CFU mL^{-1}, 10^6 cells mL^{-1}) and exposed to simulated sunlight irradiation (W-W or W-W2 + H_2O_2 + sunlight). These bacteria concentrations were chosen in order to achieve their easy monitoring. Further, samples were taken at different times. Dark (without irradiation in presence of 10 mg L^{-1} of H_2O_2) (W-W or W-W2 + H_2O_2 + Dark) and photolysis (W-W or W-W2 + sunlight) experiments (under simulated sunlight irradiation

in absence of H_2O_2) were also performed. The H_2O_2 concentration of 10 mg L^{-1} was found by several reported studies as optimal to achieve an efficient bacteria inactivation in water through photo-Fenton process (Alvear-Daza et al., 2018b, 2018a; Gutiérrez-Zapata et al., 2017a; Sciacca et al., 2010; Spuhler et al., 2010).

Post-irradiation effects were evaluated leaving the water after the photochemical treatment in presence or absence (by adding sodium thiosulfate) of residual H_2O_2 under dark conditions for 24 h. Viability was further measured (more details about this methodology is reported in supplemental information).

2.6. Morphology of bacteria cells before and after photochemical treatment

E. coli K-12 and *Klebsiella pneumoniae* morphology was followed by using a scanning electron microscope (SEM) (Jeol JSM6490LV) on a microscope slide with 6 mm wells, loaded with 20 μL of fixed sample, initially dehydrated and later metalized with gold. Electronic Micrographs were taken on every sample collected during the experiments with natural well water samples irradiated with simulated solar light (300 W m^{-2}) in presence of 10 mg L^{-1} of H_2O_2.

3. Results and discussion

3.1. Simultaneously monitoring of viability (DVC-FISH) and culturability (plate count-PC) during bacteria inactivation in groundwater samples

Physical-chemical characterization of well water samples (W-W) was performed exhibiting presence of nitrates, fluoride, iron, carbonates, phosphates, chloride, dissolved organic matter (DOM), and neutral pH (W-W in Table 1). Results showed that *E. coli* strain underwent a complete loss of culturability after 60 min of simulated sunlight irradiation (Fig. 1a) while *K. pneumoniae* needed 120 min (Fig. 1c) (W-W+H_2O_2 + sunlight (PC)). Experiments in presence of H_2O_2 but without sunlight irradiation (W-W+H_2O_2 Dark (PC)) exhibited culturability decreasing of 3.5- and 3-folds for *E. coli* and *K. pneumoniae* respectively in 140 min, while experiment photolysis (well water samples exposed to sunlight irradiation without H_2O_2) (W-W + sunlight (PC)) showed culturability decreasing of 4.5- and 6-folds to *E. coli* and *K. pneumoniae* respectively in the same time. In contrast, when viability by DVC-FISH was evaluated in experiments in presence of H_2O_2 and simulated sunlight irradiation (W-W+H_2O_2 + sunlight (V)), it was necessary 360 min of irradiation to achieve the complete viability reduction for *E. coli* while *K. pneumoniae* only reached a decreased of viability of 3-logs after the same irradiation time. Temperature in all experiments oscillated between 35 and 38C. Fig. 1b and d show the DVC-FISH pictures for *E. coli* and *K. pneumoniae* respectively for W-W+H_2O_2 + sunlight (V) experiments taken by epifluorescence microscopy where viable bacteria exhibit a red color.

Table 1
Physical-chemical characterization of well water samples.

Parameter	W-W	W-W2
pH	7.01	7.24
Total Iron (mg L^{-1})	0.390	0.303
Bicarbonate (mg L^{-1})	278	264
Phosphate (mg L^{-1})	0.24	0.17
Fluoride (mg L^{-1})	1.23	1.00
Chloride (mg L^{-1})	25	30
Nitrates (mg L^{-1})	1.63	1.45
Color (PCU)	0.5	0.3
Turbidity (NTU)	0.5	0.7
Dissolved organic carbon (DOC) (mg L^{-1})	2.34	2.88

Hydrogen peroxide, total iron, ferrous ion concentrations and pH evolution were monitored during experiments with *E. coli* and *K. pneumoniae* (Fig. 2a and b respectively). For experiments with both strains, there was a pH increasing from 7.2 to 8.1 after 360 min of simulated sunlight irradiation. On the other hand, H_2O_2 was partially consumed in W-W+H_2O_2 + sunlight experiments leaving a residual concentration of 1.56 and 2.10 mg L^{-1} to *K. pneumoniae* and *E. coli* respectively, after 360 min of sunlight irradiation. Dark experiments exhibited also residual H_2O_2 concentrations for *E. coli* and *K. pneumoniae* near to 5 mg L^{-1}. Total iron concentration decreased in irradiated and dark experiments; however, the highest diminution (~75%) was observed in sunlight-irradiated well water samples in presence of hydrogen peroxide. Regarding ferrous ion, this was also naturally present in water samples at concentrations around 0.05 mg L^{-1} and this rose slightly during experimental conditions (until ~0.1 mg L^{-1}). The depletion of total iron concentration has been reported (Ruales-Lonfat et al., 2014; Zapata et al., 2010), and it can be explained by several events such as iron sticking on magnetic stirring bars and reactor walls, iron precipitation induced by temperature rising and bacteria iron sequestration.

Otherwise, rising of ferrous iron concentration observed in these experiments could be due to photoreduction reactions occurring in iron (hydr)oxides surfaces (heterogeneous photo-Fenton reactions) and ferric-DOM species (Eq. (1)) and eventually releasing of ferrous ions adsorbed onto inosilicate. Ruales-Lonfat et al., (Ruales-Lonfat et al., 2014) have also suggested the participation of solar irradiated ferric-siderophore complexes in bacteria inactivation through a similar LMCT mechanism described in Eq. (1) where ferrous ion is produced. This fact is supported by Spuhler et al., where it was described a strong iron sequestration by *E. coli* cells in Milli-Q water (Spuhler et al., 2010).

$$\left[Fe^{3+}L\right] + h\nu \rightarrow \left[Fe^{3+}L\right]^{*} \overset{(LMCT)}{\rightarrow} Fe^{2+}\ L^{\cdot -} \quad (1)$$

Furthermore, the participation of dissolved Fe in the viability reduction of *E. coli* and *K. pneumoniae* cells was evaluated in well water samples filtered by 0.22 µm membranes in presence of H_2O_2 10 mg L^{-1} under simulated sunlight irradiation (W-W+H_2O_2 + sunlight (V) Filt) (Fig. 1). As it was observed, viability decreasing was smaller, around 4-logs for *E. coli* while for *K. pneumoniae* was around 1-log after 360 min of irradiation showing the role of dissolved iron on bacteria inactivation. Water filtration generated a total iron concentration reduction of almost 75% since its concentration dropped from 0.390 to 0.100 mg L^{-1} while pH underwent the same behavior observed in unfiltered samples (from 7.2 to 8.1). SEM micrographs with EDS mapping of solid recovered by filtration (Fig. 3) revealed the presence of potassium, manganese, silicon, aluminum, sodium, calcium, iron, magnesium, titanium, carbon, barium and phosphorous. The solid matter contained in groundwater can also be present in form of inosilicates (pyroxenes and amphiboles) and iron (hydr)oxides such as goethite (α-FeOOH) and lepidocrocite (γ-FeOOH).

In this case, presence of pyroxenes ($(XYSi, Al)_2O_6$), where X= Ca, Na, Fe^{2+}, Mg, Mn, Zn, Li and Y= Cr, Al, Fe^{3+}, Mg, Ti, Mn) and amphiboles ($A_{0\text{-}1}B_2C_5T_8O_{22}(OH, F, Cl)$ where A= Na, K; B= Na, Zn, Li, Ca, Mn, Fe^{2+}Mg; C= Mg, Fe^{2+}, Mn, Al, Fe^{3+}, Ti, Zn, Cr and T= Si, Al, Ti, was confirmed by elements detected in SEM-EDS measurements (Fig. 3). These species exhibit an interesting property to adsorb ferrous ions that can be released to water under agitation. Precisely, Xie et al., have argued that ferrous ions released from inosilicates and iron(hydr)oxides naturally present in natural well water samples by mechanical mixing could react with molecular oxygen generating ferric ions and $O_2^{-\bullet}$ radicals (Eq. (2)) (Xie et al., 2020). These latter can undergo further disproportion reactions leading to the generation of H_2O_2 (Eq. (3)) which can participate

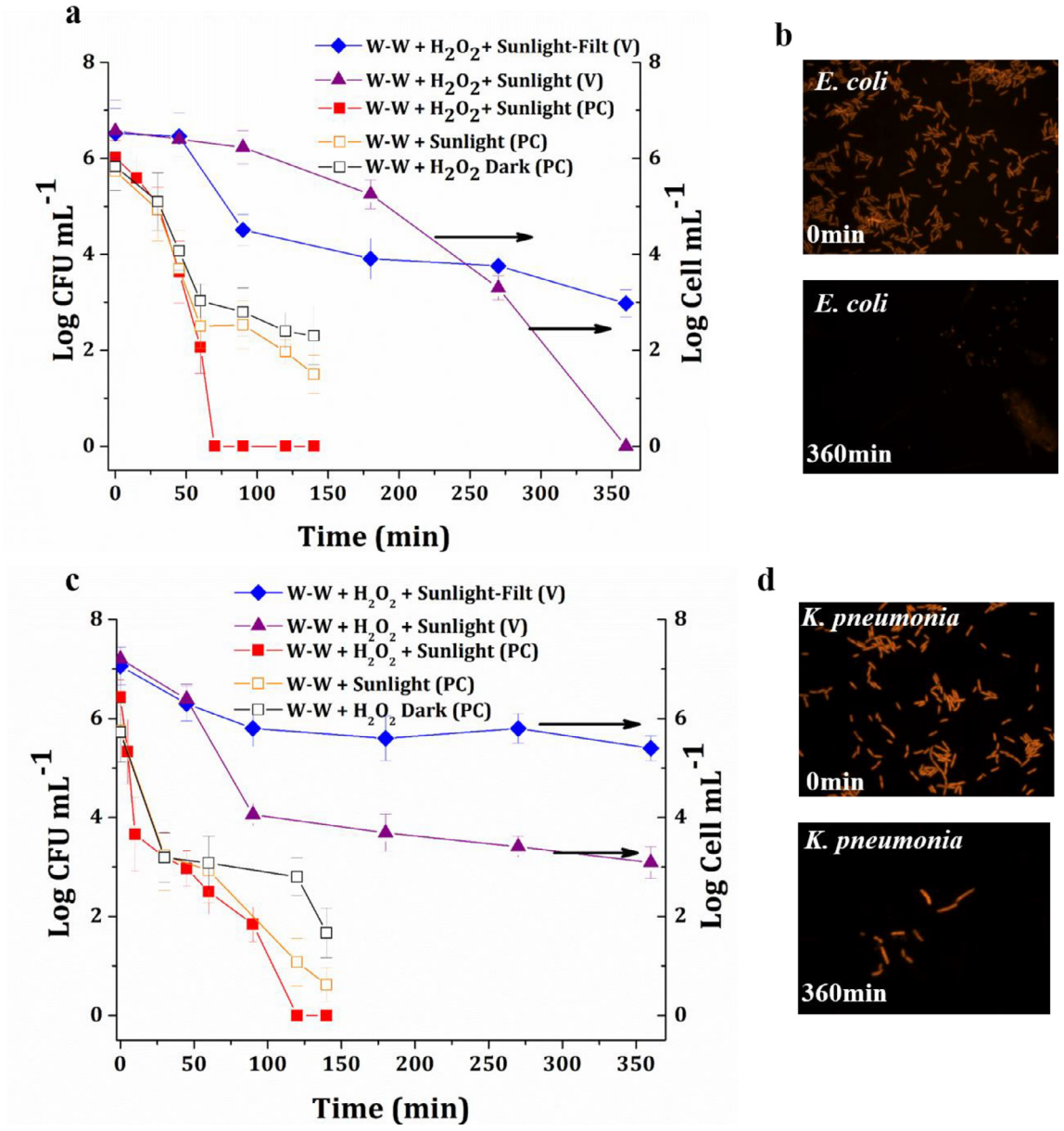

Fig. 1. Inactivation of (a) *E. coli* and (c) *Klebsiella pneumoniae* followed by culturability (PC) and viability (V) in natural well water samples containing natural total iron concentrations of 0.390 mg L^{-1}, neutral pH (7.01) under simulated sunlight irradiation (300 W m^{-2}) in presence of 10 mg L^{-1} of H_2O_2 (W-W+H_2O_2 + sunlight). Control experiments under sunlight irradiation and in absence of H_2O_2 (W-W + sunlight) and dark experiments (W-W+H_2O_2 Dark). Final temperature in all experiments oscillating between 35 and 38C. Images obtained by epifluorescence optical microscopy of (b) *E. coli* (d) *K. pneumoniae* at different treatment times.

in Fenton reaction generating $^{\bullet}$OH and ferric ions (Eq. (4)). This latter undergoing fast precipitation as iron (hydr)oxides (FeOOH).

$$Fe^{2+} + O_2 \rightarrow Fe^{3+}OOH_{(s)} + O_2^{-\cdot} \quad (2)$$

$$O_2^{-\cdot} + O_2^{-\cdot} \rightarrow O_2 + H_2O_2 \quad (3)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+}OOH_{(s)} + {}^{\cdot}OH + H^+ \quad (4)$$

On the other hand, iron (hydr)oxides as goethite and lepidocrocite have been successfully evaluated in the photocatalytic inactivation of bacteria (Ruales-Lonfat et al., 2015). These FeOOH species may exhibit photocatalytic activity leading to the generation of reactive oxygen species (ROS) such as $^{\bullet}$OH radicals, which could be enhanced by the presence of fluoride (F^- is also naturally present in groundwater at concentrations of 1.0 and 1.3 mg L^{-1}) (Du et al., 2008). We found that fluoride naturally present into groundwater could exert a positive role in bacteria inactivation and pollutants removal (H.M. Gutiérrez-Zapata et al., 2017b, 2017a). Moreover, FeOOH species in presence of H_2O_2 can also contribute in the generation of $^{\bullet}$OH radical through heterogeneous dark Fenton reactions and photo-Fenton reactions (Ruales-Lonfat et al., 2015).

Other species as nitrates, nitrites and bicarbonates also play an important role in photoinduced chemical reactions leading to the generation of $^{\bullet}$OH radicals. Nitrates, which are present in groundwater samples (1.63 or 1.45 mg L^{-1}), under UV-B (280–315 nm) irradiation induce n→ π^*transitions yielding $^{\bullet}$OH radicals and NO_2^-. This latter under UV-A irradiation (315–400 nm) can also generate hydroxyl radical at neutral pH (Vione and Scozzaro, 2019):

$$NO_3^- + hv \rightarrow {}^{\cdot}NO_2 + O^{-\cdot} \left(O^{-\cdot} + H_2O \leftrightarrow {}^{\cdot}OH + OH^-\right) \quad (5)$$

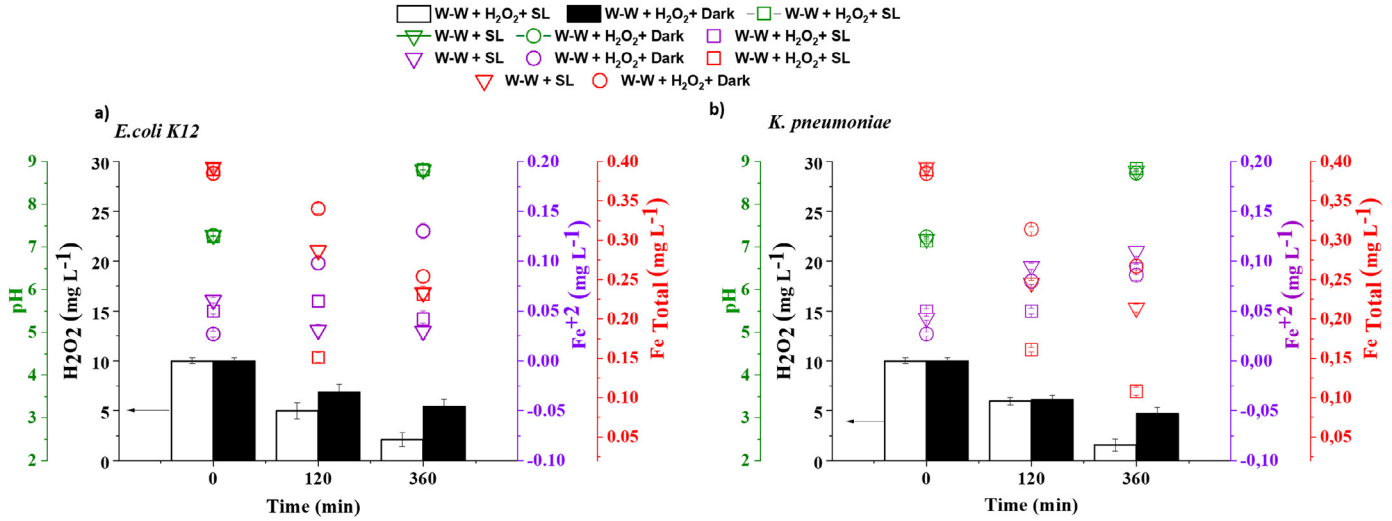

Fig. 2. Total iron, ferrous ion, H_2O_2 concentrations, and pH followed during illuminated (W-W+H_2O_2 + sunlight and W-W + sunlight) and dark experiments (W-W+H_2O_2 Dark) of (a) *E. coli* and (b) *K. pneumoniae* inactivation in well water samples containing initial natural total iron concentrations of 0.390 mg L^{-1} at neutral pH (7.01). Intensity of simulated sunlight irradiation was 300 W m^{-2}.

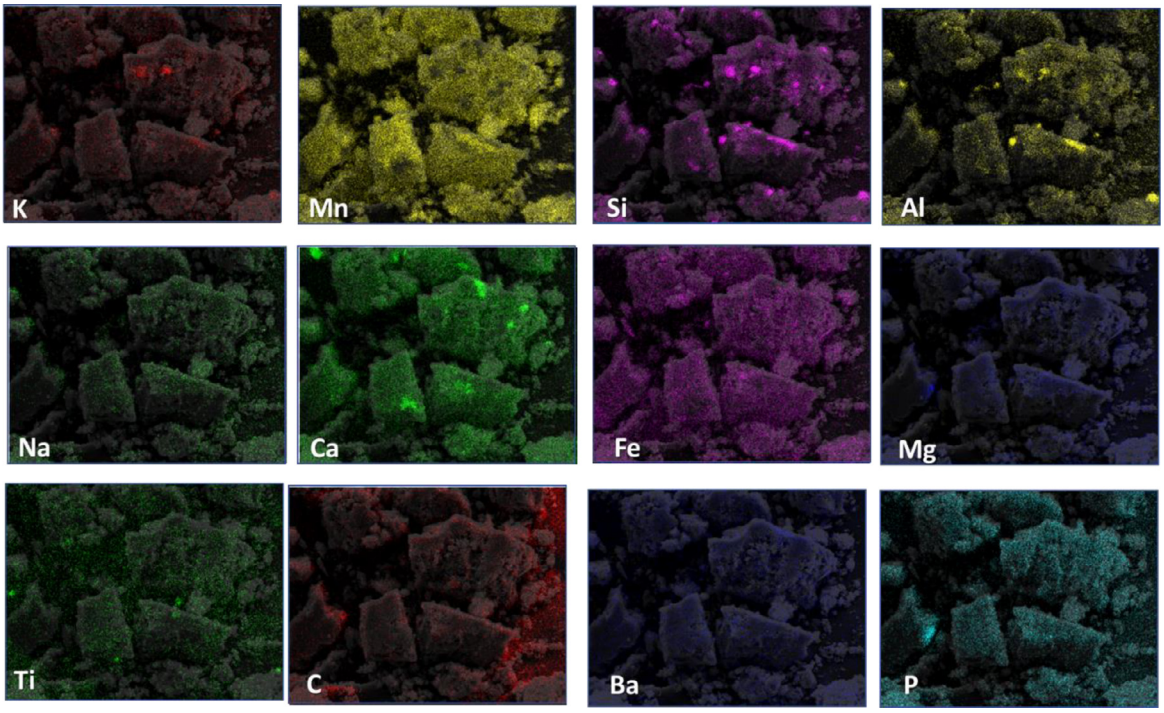

Fig. 3. SEM micrographs and EDS mapping of solids recovered from well water samples. Line Kα1 of C, Si, Mn, Fe, K, Al, P, Na, Ca, Mg, Ba, and Ti elements.

$$NO_3^- + hv \rightarrow NO_2 + O^{\cdot} \quad (6)$$

$$NO_2^- + hv \rightarrow {}^{\cdot}NO + O^{-\cdot} \quad (7)$$

$$O^{-\cdot} + H^+ \rightleftharpoons {}^{\cdot}OH \quad (8)$$

Ndounla et al. (Ndounla et al., 2014; Ndounla and Pulgarin, 2014) have also suggested the potential role of nitrates and nitrites by photoinducing $^{\bullet}$OH radicals and causing bacterial abatement in solar irradiated Sahelian groundwater samples in presence of H_2O_2.

Regarding the dissolved organic matter, groundwater herein studied did not show color often attributed to presence of humic acids of high molecular weight (chromophoric dissolved organic matter-CDOM) (Mostofa et al., 2013), so it is possible suggesting that DOM in these samples could be composed by low molecular weight substances such as formiates and acetates which may come from anaerobic-biogeochemical processes occurring naturally in well water samples (Appelo and Postma, 2005). Formiates and acetates can form photoactive (under UV and visible light) and high soluble complexes with ferric ions at circumneutral pH leading to the generation of ferrous ions and oxidized ligand (Eq. (1)) (Ruales-Lonfat et al., 2016). The former reacts with H_2O_2 through the classical Fenton reaction yielding $^{\bullet}$OH radicals (Eq. (4)) while the latter reacts with molecular oxygen leading to the generation of superoxide anion radical ($O_2^{-\bullet}$) (Eq. (9)). Superoxide anion radical (pK_a= 4.8) is unstable in aqueous media undergoing dispro-

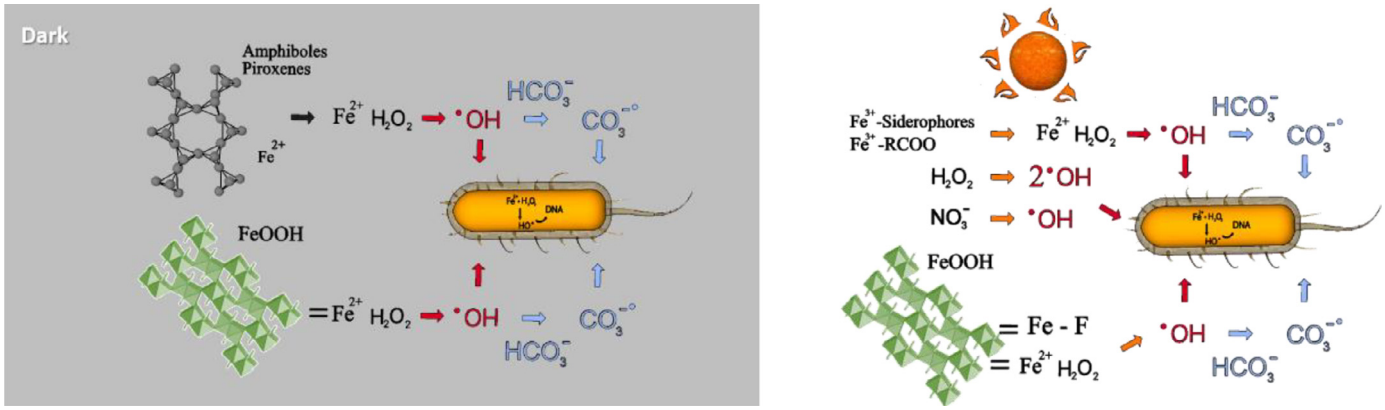

Fig. 4. Suggested mechanism of dark and sunlight photo-induced processes responsible of $^{\bullet}OH$ and $CO_3^{-\bullet}$ radicals formation occurring in well water samples containing 10 mg L^{-1} of H_2O_2.

portion reactions and finally producing hydrogen peroxide (Eq. (3)) (Sawyer and Valentine, 1981).

$$L^{-\cdot} + O_2 \rightarrow O_2^{-\cdot} + L_{Ox} \quad (9)$$

Water samples also exhibited the presence of bicarbonates (HCO_3^-) at concentrations oscillating between 264 and 278 mg L^{-1}. Bicarbonates can react with $^{\bullet}OH$ radicals leading to the generation of a less oxidative carbonyl radical ($CO_3^{-\bullet}$ 1.7 V vs NHE) (Eq. (10)) (Dell'Arciprete et al., 2012; Rommozzi et al., 2020). This radical could have a longer lifetime than $^{\bullet}OH$ being more selective toward oxidation of external cellular membranes.

$$HCO_3^- + HO^{\bullet} \rightarrow CO_3^{-\bullet} + H_2O \; k = 8.5 \; x \; 10^6 M^{-1} s^{-1} \quad (10)$$

Dell'Arciprete et al., (Dell'Arciprete et al., 2012) have argued that $CO_3^{-\bullet}$ radicals can participate in both electron-transfer and hydrogen-abstraction reactions producing the oxidation of organic substances in water. On the other hand, Wolcott et al., (Wolcott et al., 1994) and Rommozzi et al., (Rommozzi et al., 2020) have suggested the participation of $CO_3^{-\bullet}$ radicals in bacteria inactivation.

The increasing of pH during simulated sunlight photoinduced reactions (W-W+H_2O_2 + sunlight) was also observed by Ndounla et al., Ndounla et al., 2014) and our previous studies (H.M. Gutiérrez-Zapata et al., 2017b) in the bacteria abatement by photochemical reactions involving presence of H_2O_2 in Sahelian and Colombian well water samples. Degradation of cell biomolecules, alteration of CO_2-carbonate equilibrium and CO_2 degassing by raising of water temperature and photochemical reactions involving NO_3^- (Eqs. (5)–((8)), could be responsible of this event.

On the other hand, bacteria inactivation mechanisms by sunlight photoinduced or dark H_2O_2 processes, have been widely reported in studies about how bacteria inactivation could be achieved. For instance, addition of H_2O_2 without light irradiation could lead to the bacteria inactivation. At H_2O_2 concentrations higher than 1 mM (34 mg L^{-1}), damages to cellular macromolecules can occur (Imlay, 2009; Uhl et al., 2015). Since H_2O_2 is an uncharged molecule, this could easily cross the external membrane. Once hydrogen peroxide is present into the cell, catalase enzymes can act degrading it. However, if its concentration is higher than the cell antioxidant capacity, hydrogen peroxide can produce detrimental effects on several macromolecules, moreover, it could generate intracellular Fenton reactions with ferrous anions also present yielding $^{\bullet}OH$ radicals which may attack DNA molecules. However, bacteria could not be sensible to initial concentrations of H_2O_2 used herein as it has already reported by Spuhler et al., (Spuhler et al., 2010) where it was demonstrated that 10 mg L^{-1} of hydrogen peroxide in Milli-Q water did not exhibit an important inactivation of *E. coli* K-12 cells. Moreover, Rodriguez-Chueca et al. (Rodríguez-Chueca et al., 2014) have also reported that *E. coli* and *Enterococcus faecalis* were not strongly affected by H_2O_2 concentrations of 50 mg L^{-1}.

Both UV-B and UV-A coming from sunlight participate in bacteria inactivation. UV-B light can produce detrimental effects on DNA, damaging molecules of pyrimidine bases and siderophores as enterobactin releasing intracellular iron while UV-A can affect catalases (Cat) and superoxide dismutase (SOD) (Giannakis et al., 2016; Santos et al., 2012). Since SOD and Cat activities can be strongly reduced under sunlight irradiation, intracellular regulation of H_2O_2 and superoxide radical ($O_2^{-\bullet}$) could be strongly affected. $O_2^{-\bullet}$ may react with Fe/S clusters oxidizing them and releasing intracellular ferrous ions which in presence of H_2O_2 can lead to the intracellular Fenton reactions.

In resume, we suggest that enhanced dark and photoinduced events by H_2O_2 addition and colloidal matter (iron (hydr)oxides and inosilicates), and dissolved organic and inorganic species (formiates- and citrates-ferric complexes, nitrates, nitrites, and bicarbonates) naturally present in well waters would be responsible of $^{\bullet}OH$ radical formation. Reaction of $^{\bullet}OH$ radicals with bicarbonates may induce the generation of $CO_3^{-\bullet}$ species and thus, both transient species could be responsible of bacteria inactivation (Fig. 4). Hydroxyl radicals have short lifetimes (nanoseconds) and high oxidative power (~2.7 V vs NHE) participating in the lipid peroxidation of cell membranes (Cheng and Li, 2007) while it has also been described the possible participation of $CO_3^{-\bullet}$ radicals in damages of bacteria external membranes (Wolcott et al., 1994).

3.2. Morphological changes of bacterial cells after exposure to H_2O_2 10 mg L^{-1} and sunlight irradiation by SEM

Figs. 5 and 6 revealed morphological characteristics of *E. coli* and *K. pneumoniae* cells respectively taken at different irradiation times in experiments where H_2O_2 and simulated sunlight irradiation were simultaneously present (W-W+H_2O_2 + sunlight). Cell shape changes were observed at intermediate irradiation times of 60 and 120 min for *E. coli* and *K. pneumoniae* respectively. Cells underwent shape changes from bacillus to coccus (red circles) and size reduction. However, after 360 min of sunlight irradiation, it is evident that *E. coli* cells were more strongly affected than *K. pneumoniae* since the former exhibited important damages localized on the external membrane (arrows in Figs. 5 and 6).

As it was described, the simple addition of hydrogen peroxide to sunlight irradiated-well water samples led to a culturability decreasing to both bacteria strains. However, when viability cell was evaluated by DVC-FISH, bacteria inactivation did not follow the same behavior since it was necessary for *E. coli* 6-fold more time to

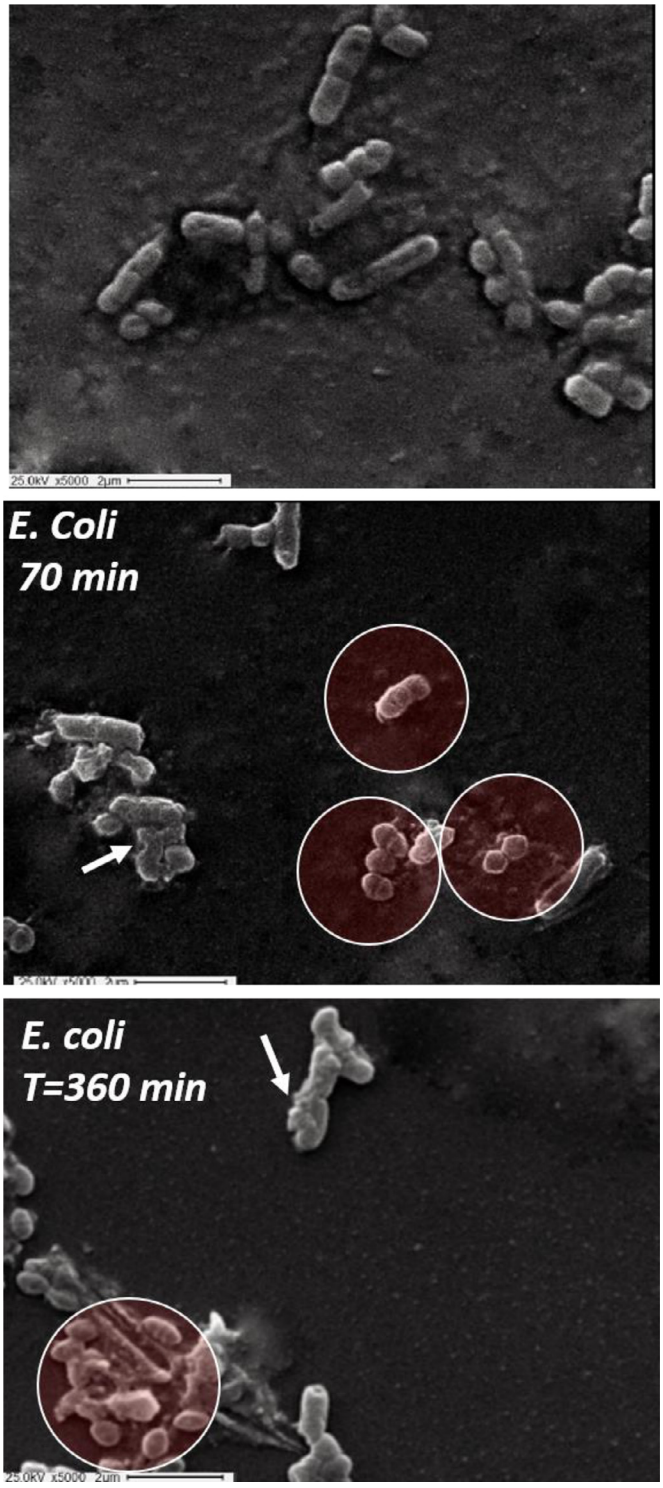

Fig. 5. SEM micrographs of *E. coli* cells during W-W+H_2O_2 + sunlight experiments carried out in natural well water samples containing initial natural total iron concentrations of 0.390 mg L^{-1} at neutral pH (7.01). Intensity of simulated sunlight irradiation was 300 W m^{-2}.

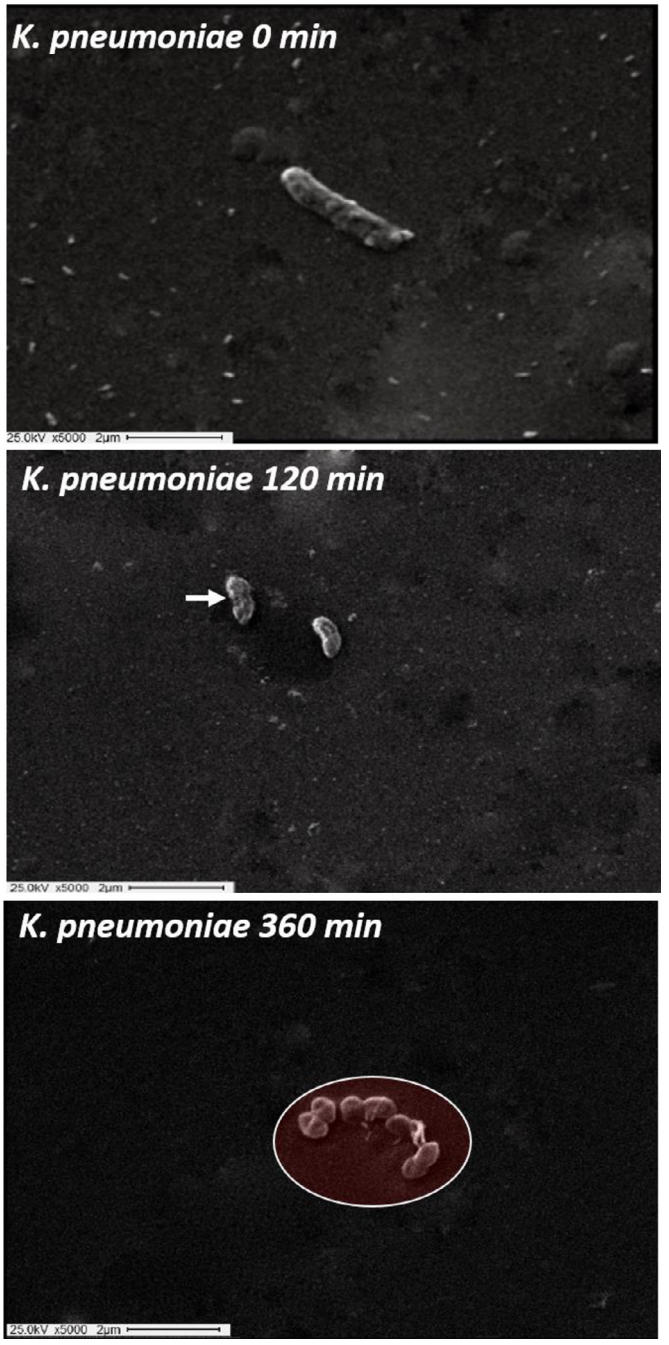

Fig. 6. SEM micrographs of *K. pneumoniae* cells during W-W+H_2O_2 + sunlight experiments carried out in well water samples containing initial natural total iron concentrations of 0.390 mg L^{-1} at neutral pH (7.01). Intensity of simulated sunlight irradiation was 300 W m^{-2}.

achieve total viability reduction while *K. pneumoniae* viability was not never totally reduced.

Recently, it has been highlighted that bacteria under oxidative stress can enter into a starvation mode of metabolism called viable but non-culturable state (VBNC) where bacteria are unable to grow using conventional culture media (Chen et al., 2018; Kim et al., 2018). Gram negative bacteria possess a protein called OxyR which regulates the expression of several genes whose main role is linked to the antioxidative response controlling mainly the peroxidases synthesis (*katG*), induction of VBNC states and iron homeostasis (*fur*) (Cornelis et al., 2011; Ding et al., 2017; Kim et al., 2018). We suggest that at short irradiation times (60 or 120 min), both bacteria strains underwent oxidative stress caused by H_2O_2-photoinduced events in natural well water samples inducing VBNC states since SEM micrographs revealed both morphological changes and cell size reduction either in *E. coli* or *K. pneumoniae* cells compatible with VBNC states. Kim et al., (Kim et al., 2018) reported that *E. coli* K12 cells under oxidative stress exhibited a morphological change from the typical bacillus shape to coccus while Wei

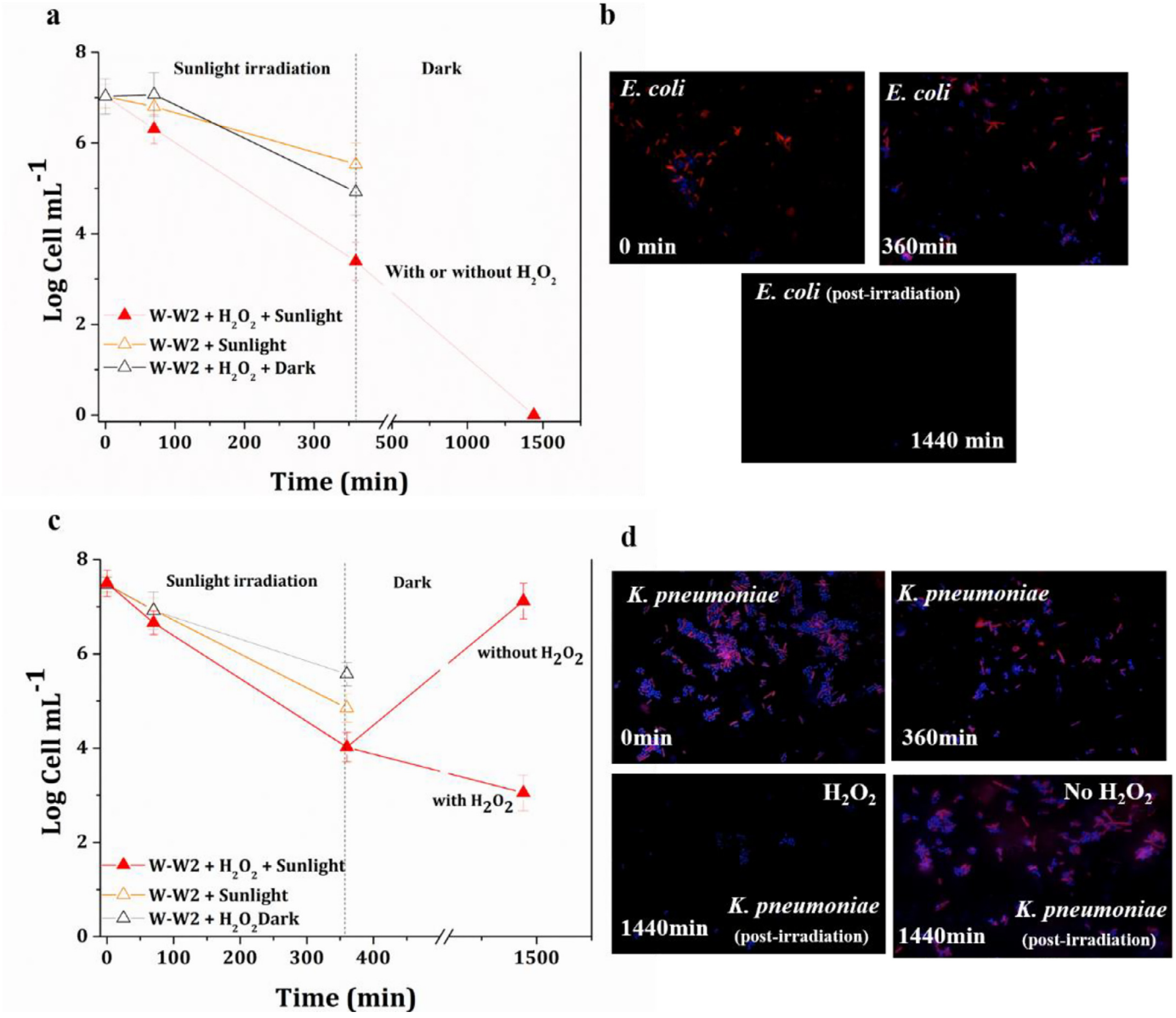

Fig. 7. Inactivation of (a) *E. coli* and (c) *Klebsiella pneumoniae* followed by viability (V) in natural well water samples (W-W2) containing natural total iron concentrations of 0.303 mg L^{-1}, neutral pH (7.24) under simulated sunlight irradiation (300 W m^{-2}) in presence of 10 mg L^{-1} of H_2O_2 (W-W2 + H_2O_2 + sunlight). Control experiments under sunlight irradiation and in absence of H_2O_2 (W-W2 + sunlight) and dark experiments (W-W2+ H_2O_2 Dark). Final temperature in all experiments oscillating between 35 and 38°C. Images obtained by epifluorescence optical microscopy of (b) *E. coli* (d) *K. pneumoniae* at different treatment times.

et al., (Wei and Zhao, 2018) studied cells size decreasing in VBNC *E. coli* O157:H7 cells.

Results of viability and morphological changes determined by SEM (Fig. 6), showed that *Klebsiella pneumoniae* evidenced an important resistance to inactivation compared to *E. coli* although some studies have reported by following only bacteria culturability that photo-Fenton process is highly effective inactivating any kind of bacteria strain (Giannakis et al., 2018; Serna-Galvis et al., 2019). During millions of years, bacteria have developed metabolic processes to protect themselves from oxygen which could lead to the intracellular generation of reactive oxygen species (ROS) such as superoxide ($O_2^{-\bullet}$) and H_2O_2 by using SOD and Cat enzymes, respectively. Moreover, extracellular and intracellular iron regulation by siderophores and Fe-S cluster proteins is also important since simultaneous presence of ferrous iron and H_2O_2 into the cell could lead to the intracellular Fenton reaction yielding $^{\bullet}$OH radicals which could attack DNA causing lethal damages to the microorganism (Imlay, 2009). Some studies have shown that siderophores could protect bacteria from oxidative stress regulating the iron concentration and avoiding intracellular Fenton reaction (Behnsen and Raffatellu, 2016; Zhang et al., 2017).

Bacteria highly resistant and virulent such as *K. pneumoniae* have developed strategies to resist the host's defenses (Paczosa and Mecsas, 2016). *Klebsiella pneumoniae* exhibits three different catalase enzymes instead two frequently found in *E. coli*, this fact could grant to this bacterium a higher capacity to resist oxidative stress (Goldberg and Hochman, 1989). On the other hand, *Klebsiella pneumoniae* exhibits a higher capacity to sequester iron since some strains possess at least 10 putative iron-uptake systems (Clegg and Murphy, 2016). This bacterium secretes siderophores such as aerobactin and enterochelin. Precisely, this latter has been identified as a protector agent against oxidative stress in *S. typhimurium* strains (Achard et al., 2013). On the other hand, OxyR regulon in *K. pneumoniae* has been related with high virulence, high re-

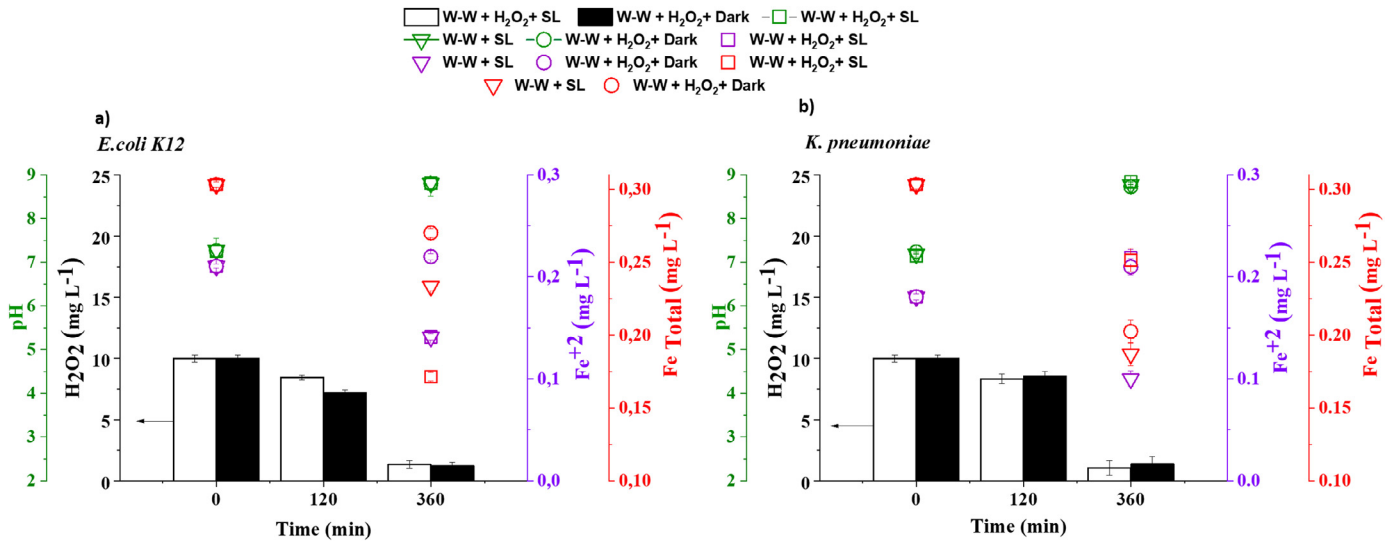

Fig. 8. Total iron, ferrous ion, H_2O_2 concentrations, and pH followed during illuminated (W-W2 + H_2O_2 + sunlight and W-W2 + sunlight) and dark experiments (W-W2+ H_2O_2 Dark) of (a) *E. coli* and (b) *K. pneumoniae* inactivation in well water samples (W-W2) containing initial natural total iron concentrations of 0.303 mg L^{-1} at neutral pH (7.24). Intensity of simulated sunlight irradiation was 300 W m^{-2}.

sistance towards oxidative stress, and synthesis of polysaccharide layer (Hennequin and Forestier, 2009; Srinivasan et al., 2013). Another important point is related with morphological differences between *E. coli* and *K. pneumoniae*. This latter exhibits a prominent polysaccharide layer which could offer more protection against the oxidative attack of extracellular photoinduced radical species such as $^{\bullet}OH$ and $CO_3^{-\bullet}$ (Paczosa and Mecsas, 2016). A previous study reported high resistance of *Enterococcus faecalis* to photo-Fenton treatment of wastewaters and authors related this resistance to high capacity of bacterium to control iron homeostasis (Rodríguez-Chueca et al., 2014). This higher resistance could be in agreement with the minor damage observed by SEM after 360 min of sunlight irradiation and presence of H_2O_2 (Fig. 6).

3.3. Study of post-irradiation effects (with or without residual H_2O_2) on bacterial growth monitoring of viability (DVC-FISH)

Physical-chemical characteristics of W-W2 samples are showed in Table 1. This time, total iron concentration was 0.303 mg L^{-1} while presence of fluoride, carbonates, DOM, pH was similar than those reported in Section 3.1.

Photolysis (W-W2 + sunlight) and dark experiments in presence of hydrogen peroxide (W-W2 + H_2O_2 dark) exhibited viability reductions of 1.5–2.0 logs for both *E. coli* and *K pneumoniae* after 360 min of irradiation respectively. Simultaneous presence of H_2O_2 and sunlight irradiation (W-W2 + H_2O_2 + sunlight) were ineffective to obtain a complete viability reduction in 360 min of irradiation (Fig. 7).

The pH variation during the experiment was also followed and the same pattern observed in Section 3.1 was obtained for *E. coli* and *K. pneumoniae* (Fig. 8a and b respectively). Total iron and ferrous ion concentrations revealed an interesting behavior. For instance, initial ferrous ion concentration was higher this time (0.100 mg L^{-1}) and its concentration increased slightly in dark experiments (W-W2 + H_2O_2 dark) and under simultaneous presence of sunlight and hydrogen peroxide (W-W2 + H_2O_2 + sunlight) (Fig. 8a and b). Conversely, total iron concentration dropped in all experiments. Concentration of hydrogen peroxide dropped strongly in W-W2 + H_2O_2 dark and W-W2 + H_2O_2 + sunlight experiments reaching values around 1–1.3 mg L^{-1} after 360 min as seen in Fig. 8.

Post-irradiation effects were evaluated in presence and absence of sodium thiosulfate (to eliminate residual H_2O_2 concentrations ~ 1.3 and 1 mg L^{-1} for *E. coli* and *K. pneumoniae* respectively). As it can be seen in Fig. 7, *E. coli* viability kept decreasing in dark conditions either in presence or absence of H_2O_2. In contrast, *K. pneumoniae* underwent 1-log reduction in presence of remaining H_2O_2, however, when hydrogen peroxide was eliminated from water, this bacterium recovered its initial population as it was observed in epifluorescence optical microscopy pictures (Fig. 7d).

Therefore, it was found by viability measurements that *K. pneumoniae* was more resistant than *E. coli* toward post-irradiation effects. This latter term was coined by Rincon and Pulgarin in 2004 (Rincón and Pulgarin, 2004). They described the post-irradiated dark phenomena occurring in bacteria cells exposed to either photocatalytic or photo-Fenton processes where cells can recover or not their culturability. Authors argued that recovering of bacteria culturability could be due to dark repair mechanisms of injured cells after the photochemical processes. In 2006 the same authors reported post-irradiation effects in systems Fe^{3+}/H_2O_2/simulated sunlight irradiation, where *E. coli* culturability kept decreasing in dark periods due possibly to an extracellular Fenton process occurring with residual H_2O_2 (Rincón and Pulgarin, 2006). We found here the same behavior for *E. coli* cells probably suggesting that $^{\bullet}OH$ radical attack during irradiated experiments caused an important damage on external membrane of cell, as it was observed by SEM micrographs (Fig. 5), altering its semipermeability and leaving the free pass of extracellular H_2O_2. Moreover, residual hydrogen peroxide in presence of ferrous ions (which are also present after the photochemical process) could also induce extracellular Fenton reactions keeping the damage on external membrane as it was also described by Ndounla et al., (Ndounla et al., 2014). On the other hand, UV-B and UV-A irradiation could be responsible as well of catalase and superoxide dismutase inactivation and intracellular ferrous ion releasing. All these events may allow the production of intracellular Fenton reaction causing lethal damage on the microorganism as outlined in Fig. 9. Meanwhile, *K. pneumoniae* was more resistant since its stronger antioxidant capacity may allow a better defense against photoinduced ROS and further extra- and intracellular-Fenton reactions by controlling the iron homeostasis and having a best H_2O_2 depletion system than *E. coli* cells as described above.

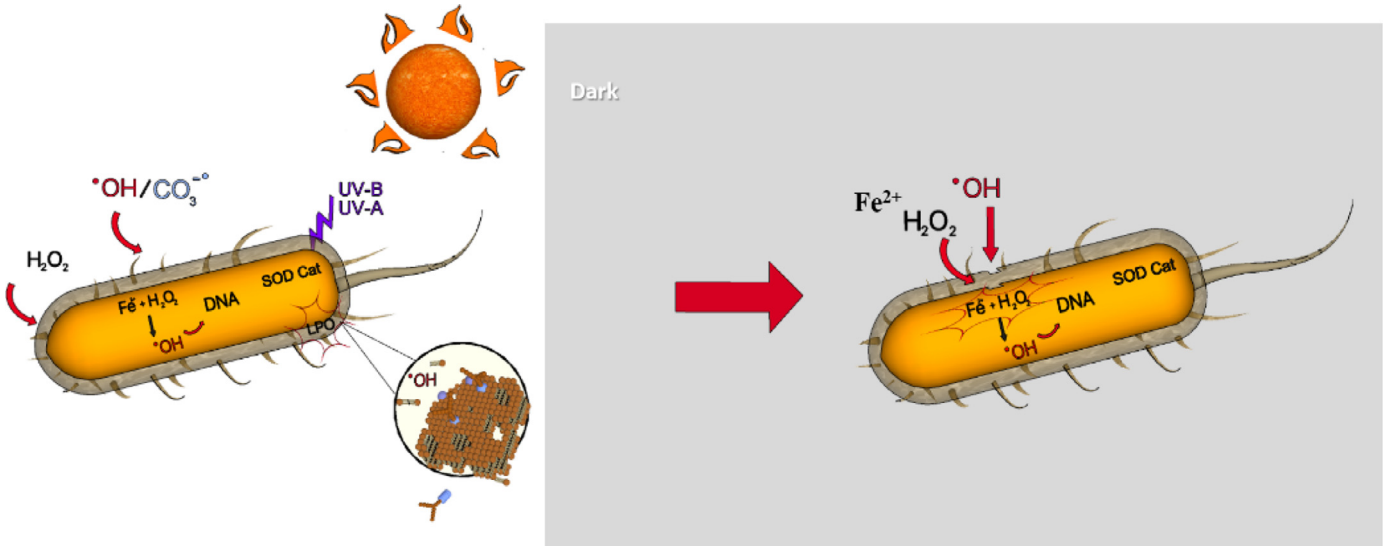

Fig. 9. Dark post-irradiation events occurring by the simultaneous presence of remaining H_2O_2 concentrations responsible of bacteria inactivation in natural well water samples.

Regarding the presence of residual H_2O_2 and its possible impact on humans, concentrations below 20–50 μM (0.7–1.7 mg L^{-1}) exhibit a limited cytotoxicity to many cell types (Halliwell et al., 2000). In our experiments, residual concentration of hydrogen peroxide was around 2.0–1.0 mg L^{-1} which would not have an important impact. Moreover, this molecule undergoes a rapid degradation in natural waters and does not induce bioaccumulation (Institute for Health and Consumer Protection European Chemicals Bureau, 2003). Some studies have reported that remaining H_2O_2 in water coming from advanced oxidation processes can undergo a rapid degradation due to biotic and abiotic reactions (Wang et al., 2017).

4. Conclusions

Viability dropping of either *Klebsiella pneumoniae* or *E. coli* cells was never according with culturability decreasing perhaps because oxidative stress caused by photochemical and dark events, could induce to the cells into viable but non culturable (VBNC) states.

Klebsiella pneumoniae demonstrated being more resistant cell to photochemical and dark reactions probably due to its strong antioxidant capacity and morphological features which protected better the bacterium towards the attack of photoinduced ROS.

Presence of residual H_2O_2 and ferrous ions was crucial to keep the bacteria abatement in dark post-irradiation events, however, *K. pneumoniae* demonstrated to be resistant. This bacterium showed a population recovering in absence of H_2O_2 while *E. coli* cells underwent inactivation either in presence or absence of oxidant.

Finally, results revealed that natural well water samples exhibit interesting physical-chemical features to be used in solar H_2O_2-photoinduced processes to produce drinking waters. Moreover, it is highlighted that the only using of culturability procedures to evaluate the performance of water disinfection treatments based on H_2O_2/sunlight processes could be limited since induction of VBNC states can be promoted and making necessary the using of other microbiological procedures such as bacterial viability.

Declaration of Competing Interest

None

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2020.116499.

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