1 **Title:** *Legionella jordanis* inactivation ~~from~~ in water by solar driven processes: EMA-
2 qPCR versus culture-based analyses for new mechanistic insights.

3

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ABSTRACT

In this contribution, the validation of EMA-qPCR method for the quantification of viable *Legionella spp* in water after solar treatments was carried out. EMA-qPCR was used to evaluate the different effects of several solar water disinfection processes over this bacterium, and furthermore their mode of action. Inactivation of *Legionella jordanis* in water by solar photocatalytic (TiO_2 and TiO_2/H_2O_2) and solar photochemical (solar/H_2O_2 and solar disinfection) processes have been investigated under natural sunlight. Culture-based and molecular (EMA-qPCR) techniques were systematically compared for the analysis of treated water samples. Solar tests were done under natural solar radiation (clear sky) and ambient temperature (20-35ºC) for 2 hours, using H_2O_2/Solar (10, 20 and 50 mg/L), TiO_2/Solar (100, 200, 300, 400, and 500 mg/L) and TiO_2/H_2O_2/Solar (100/10, 200/10, 500/10 mg/L). According to culture-based method, the best results of bacterial inactivation were obtained for 500/10 mg/L of TiO_2/H_2O_2. The order of efficiency to reach complete inactivation was: TiO_2/H_2O_2/solar (5 min) > TiO_2/solar (15 min) ≈ H_2O_2/solar (15 min) > Solar only disinfection (90 min). Moreover, EMA-qPCR and culturable counting results showed a direct correlation for ~~those~~ samples treated with TiO_2/solar for those catalyst concentrations that generate a strong oxidative attack over the cell wall. EMA-qPCR results demonstrated to be a good method to detect damaged and dead cells when the treatment affects the integrity of the cell's membrane, as occurs under photocatalysis. Meanwhile for solar disinfection and solar/H_2O_2 (at non-toxic concentrations, <1.5 mM), where membrane integrity remained unaltered, EMA-qPCR results couldn´t discriminate between alive and dead cells, even when the bacteria were not culturable.

Keywords: EMA; *Legionella jordanis*; real time qPCR; TiO_2; hydrogen peroxide.

1. INTRODUCTION

The Gram negative bacterium *Legionella spp.* is worldwide spread in freshwater, especially man-made water systems including ~~like~~ cooling towers, hot distribution system, potable water, spa pools, fountains, etc. [1], and ~~including~~ waste water treatment plants, within the active aerobic sludge [2]. The ubiquity of this bacterium can be explained by its ability to survive in a wide variety of environmental conditions, including stressing habitats with high temperatures, a broad range of pH conditions, salt concentrations, and low availability of nutrients; they has been detected associated to biofilms or parasitism of protozoan hosts [3]. The most commonly accepted mechanism of infection in humans is by inhalation or aspiration of contaminated aerosols causing Legionnaire's disease or Pontiac fever [4]. Therefore, *Legionella* genus is considered as an opportunistic pathogen of significant public health concern. The European Centre for Disease Prevention and Control (ECDC) reported 6 941 cases of Legionnaire's disease by 28 EU Member States and Norway in 2014 with 8 % of case fatality [5]. A number of guidelines and regulations consider the risk of exposure to *Legionella spp.* through the world. In Europe, Directive 2000/54/EC [6] is used as source for others national regulations, like in Austria, Belgium, Cyprus, France, Denmark, Spain, etc. [2].

In addition, methodology for *Legionella* spp detection in water samples is a big challenge due to its difficult growth and the specific requirements for its reliable enumeration, as well as the growth of unwanted microorganisms which may obscure its identification, and the possible underestimation of bacteria by the presence of viable but non-culturable *Legionella* spp, which may occur in culture-count methodologies [7]. Traditionally, the culture-based method has been the most accepted methodology for detection of *Legionella* spp in water which main disadvantages have been addressed using specific agar formulations [8]. Nevertheless, in last decades, the development of molecular tools such as quantitative real-time PCR (qPCR), has offered a faster, more sensitive, and more specific detection of *Legionella* spp in water samples as compared to culture-based techniques. However, the main drawback of qPCR lies in its overestimation of the risk of infection (by false-positive) due to the incapability to discriminate between viable and non-viable cells when DNA persists inside cells after death [9]. Therefore, the development of a qPCR-based methodology to discriminate between alive and dead cells is of major interest for detecting viable bacteria in water.

Currently, the use of nucleic acid-binding dye ethidium monoazide bromide (EMA), in combination with qPCR technique has been demonstrated to be a good tool for selectively detecting and enumerating viable bacteria by qPCR [9-14].

A wide variety of disinfection methods such as chlorination are used to control, among others pathogens, *Legionella* spp with the aim of complying with the water quality standards for different types of water systems. Nevertheless, chlorination has some disadvantages like the well-known generation of disinfection by-products (DBPs) and their high mutagenic potential for humans [15]. Therefore, the use of alternative water disinfection methods is of high interest to reduce these drawbacks. In line with this, some Advanced Oxidation processes (AOPs) driven by natural sunlight like heterogeneous photocatalysis using Titanium Dioxide (TiO_2), and TiO_2/H_2O_2 are being proposed as new approaches for water and wastewater disinfection [16]. ~~The efficacy of AOPs lies in the generation of oxidative species, in particular hydroxyl radicals ($OH^{\bullet}$), which is the second most powerful oxidative specie after fluorine~~ The mechanism under which AOPs work lies in the generation of oxidative species, mainly hydroxyl radicals ($OH^{\bullet}$), which are the second most oxidative species after fluorine. These highly oxidative species can oxidize almost all organic compounds and inactivate a wide range of microorganisms [17]. Other~~s~~ solar-driven process based on the adding of non-toxic amounts of hydrogen peroxide (H_2O_2) and natural sunlight has ~~been~~ demonstrated to induce an accelerated inactivation of several microorganisms in water [17-20]. It is believed that the mechanism of action of this method is based on the stressing effect produced by H_2O_2 and solar photons which lead to intracellular photo-Fenton reactions with the available iron inside (LIP, Labile Iron Pool) the microbial cells [17, 21].

The main goal of this research is to validate a method based on EMA-qPCR analysis for the quantification of viable bacteria of *Legionella* spp in water. This method was simultaneously compared to standard culture (with selective media)-based quantification method. The new EMA-qPCR was used to evaluate the vulnerability of *Legionella* spp to different solar AOPs and solar-driven processes and to discriminate different types of damages over bacteria by the different ~~AOPs~~ solar processes and their mode of action. Solar processes investigated were solar disinfection, H_2O_2/Solar, TiO_2/Solar and TiO_2/H_2O_2/Solar under different concentrations of reagents and catalyst.

2. MATERIAL AND METHODS

2.1 Legionella jordanis enumeration

L. jordanis (DSM 19212T) was obtained from Leibniz-Institute DSMZ German Collection of Microorganisms and Cell Cultures. Fresh liquid cultures were prepared in LB lennox (Alfa Aesar, Germany) supplemented with *Legionella* BCYE growth (Oxoid) and incubated at 37ºC in a rotary shaking for 48 h. The bacterial stationary phase concentration was ~ 10^8 CFU/mL. Bacterial suspensions were harvested by centrifugation at 900 $\times g$ for 10 min. The bacterial pellet was re-suspended in phosphate-buffered saline (PBS) and diluted to an initial concentration of 10^6 CFU/mL. The samples taken during the experiments were enumerated using the standard plate counting method through serial 10-fold dilutions in PBS in *Legionella* CYE agar base supplemented with *Legionella* BCYE growth. Colonies were counted after incubation for 48 h at 37ºC. The detection limit (DL) was 2 CFU/mL.

2.2 DNA extraction

DNA was extracted from the pellet obtained from 1 mL of water samples using InstaGene™ Matrix (Bio Rad). DNA extraction was done according to manufacturer´s kit instructions. For EMA quantitative PCR (EMA-qPCR), before DNA extraction a pre-treatment was performed according to [9]. Briefly, 0.5 µL of EMA stock (5 mg/mL) were added to each 1 mL of water sample and kept in dark for 10 min~~utes~~. After that, samples were placed on ice and exposed to a halogen light source (Osram Lum Halostar 30/650 W) to permit photo-cross-link EMA to DNA for 15 min. Samples were then washed by centrifugation (16,000 g, 5 min) with 0.85% NaCl and immediately DNA extraction procedure was done. DNA samples were quantified using a NanoDrop spectrophotometer (NanoDrop Lite, Thermo scientific), to determine initial DNA concentration, which ranged in all cases between 1.5 and 10 ng/µL.

For qPCR and EMA-qPCR standard curve preparation, 1 mL of 10-fold dilutions ranging from 10^6 to 10^0 CFU/mL of *L. jordanis* suspension was used and the already mentioned DNA extraction procedure was performed.

2.3 Quantification by qPCR.

DNA amplifications were performed with a 7500 Fast Real Time PCR System (Applied Biosystems, USA) in 96-barcode well plates. A high priming efficiency (>95%) kit for

158 detection of *Legionella* spp (Primerdesign™ genesig® Kit for *Legionella* (all species))
159 based on 16S Ribosomal RNA gene was used according to the manufacturer's
160 instructions. The primers and probe of this kit have been designed to cover the highest
161 detection profile remaining the specificity of *Legionella* spp. Non template control
162 (Negative control) and positive control (2×10^5 Copy Number/µL) were checked to
163 validate DNA-samples amplification results. The mean cycle threshold (CT) value for
164 positive control of *Legionella* spp. (10^6 copy number/µL) was 16.02 - 16.82. In any
165 case, amplification was not observed for the negative control, ensuring reliability of
166 qPCR results and therefore no cut-offs had to be set. Each qPCR measurements for
167 every sample were done in triplicate at the same time. Each graphical point is the
168 average of the three replicates, and error bar is the corresponding standard deviation.
169 The concentration of bacteria obtained by qPCR and EMA-qPCR is given in cells
170 equivalent (CE)/mL, which was verified by quantification of the concentration of viable
171 cells, given in terms of colony forming units (CFU/mL) by plating diluted samples used
172 for standard qPCR curve.
173
174 ***2.4 Solar experiments***
175 Experiments were performed in 250 mL DURAN-glass (Schott, Germany) vessel stirred
176 reactors. Total volume of water was 200 mL with 0.0095 m^2 of irradiated surface. All
177 experiments were conducted at Plataforma Solar de Almeria (Spain, located at 37◦84N
178 and 2◦34W) under natural solar radiation on completely sunny days for 4 h (10:30–
179 14:30, local time). Distilled water was used as a reference for observation and
180 comparison of inactivation kinetics in controlled laboratory conditions, excluding the
181 contribution or interference of any other water compound. *L. jordanis* suspensions and
182 reagents added to different solar reactors were stirred at 100 rpm. Vessel reactors were
183 covered with a glass cap to allow the solar radiation entering from all directions.
184 Temperature (Checktemp, Hanna, Spain) and pH (WTW-multi720, Germany) were
185 measured directly in the reactor before and after each solar treatment. UV radiation was
186 monitored using global UVA pyranometer (300–400 nm, Model CUV5, Kipp & Zonen,
187 Netherlands) and providing data in terms of incident irradiation (W/m^2), which is the
188 solar radiant energy rate incident per unit of surface area. Four types of solar processes
189 were evaluated: (i) solar only disinfection (ii) TiO_2/solar (100, 200, 300, 400, 500
190 mg/L); (iii) H_2O_2/solar (10, 20 and 50 mg/L) and (iv) TiO_2/H_2O_2/solar (100/10, 200/10,
191 500/10 mg/L). The range of reagent and catalyst concentrations tested in this work was

192 selected based on previous works [17-22], which have non toxic effects over bacterial
193 viability. All experiments were performed in triplicate. Average of results is plotted in
194 graphs with corresponding error bars calculated as the standard deviation ~~SD~~ of the
195 results obtained from replicates.

196

197 ### *2.5 Reagents*

198 Aeroxide-P25 TiO_2 catalyst (Evonik, Germany) was used as received as slurry.
199 Hydrogen peroxide (Riedel-de Haën, Germany, 30% (w/v)) was used as received and
200 added directly into the reactor. H_2O_2 concentration was measured with a
201 spectrophotometer (PG Instruments Ltd T-60-U) at 410 nm in glass cuvettes with a 1
202 cm of path length based on the formation of a yellow complex from the reaction of
203 titanium (IV) oxysulfate (Riedel de Haën, Germany, used as received) with H_2O_2
204 following DIN 38409 H15. Absorbance was read after 5 min incubation time against a
205 H_2O_2 standard curve linear in the 0.1–10 mg/L concentration range. Ethidium
206 Monoazide Bromide (EMA) (Molecular Probes, Life Technologies, Spain) was
207 dissolved in dimethyl sulfoxide in the absence of light and stored at -20ºC.

208

209 ## 3. RESULTS AND DISCUSSION

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211 ### *3.1. Standard curve and detection limit (DL) of EMA-qPCR*

212 Standard curves of *L. jordanis* were determining through a 10-fold serial dilution of the
213 corresponding inoculum from 10^6 to 10^0 CFU/mL. A linear profile was observed over
214 6-log units. Three standard curves were performed in three different days in order to
215 determine the repeatability of the assay. Furthermore, to determine the efficiency of
216 EMA-qPCR method, qPCR analysis of standard curves with and without EMA were
217 performed simultaneously. Figure 1 shows standard curves for qPCR and EMA-qPCR.
218 Table 1 shows the average CT and CE/mL results of both types of standard curves. The
219 amplification efficiencies achieved ranged from 98% to 130% with high correlation
220 coefficient of 0.99 and 0.97 for amplification values of qPCR and EMA-qPCR,
221 respectively. Reproducibility was confirmed with ΔCT between ten-fold standard
222 dilutions of 3.11-3.09. Detection limit (DL) according to the standard curves was
223 determined in a CT value of 32.8 ± 1.9 and 35.7 ± 1.5, for qPCR and EMA-qPCR,
224 respectively. Correspondingly, the CE/mL value at the DL was found to be 3.0 ± 2.1
225 CE/mL in both cases, assuming 100% efficiency for DNA extraction (Table 1).

3.2 Thermal inactivation of L. jordanis cells

Prior to investigate the solar processes, where a solar mild-heating occurs over exposure time, we evaluated the separated effect of mid-solar temperatures reached during these processes. Therefore, the thermal effect of 45, 50 and 60 ºC on *L. jordanis* viability was investigated in darkness. The inactivation of bacteria was evaluated by standard culture method, qPCR and EMA-qPCR procedure simultaneously. Figure 2 shows the comparison between results of cells quantification using the three techniques obtained at 45 ºC (Fig. 2a), 50 ºC (Fig. 2b) and 60 ºC (Fig. 2~~3~~c). At 45 ºC, results for culture technique, EMA-qPCR and qPCR showed that viability of *L. jordanis* remains constant for 2 h, being not affected the viability of bacteria at this temperature. No significant differences among all the techniques were observed. Figure 2b shows the comparison of cells quantification using the 3 techniques for *L. jordanis* at 50 ºC. Cells counting showed 3-log reduction in 120 min. EMA-qPCR results demonstrated a reduction of 1.5-log over same time, while qPCR showed that bacteria remained viable. In the case of 60 ºC, culture&counting technique showed a complete inactivation in less than 10 min, while EMA-qPCR results showed that complete inactivation (6-log reduction) was achieved in 40 min, and qPCR showed a 4.5-log reduction of bacteria keeping a residual concentration. These results are in agreement with those reported by Delgado-Viscogliosi et al. (2009) for the inactivation of *L. pheumophila* at 70 ºC. They found that after 1h of heat treatment, viable cell counts lead to complete bacterial inactivation, qPCR (EMA) accounted for nearly 4-log units drop, while the qPCR count result remained practically constant before and after the treatment [9]. Therefore, it can be concluded that EMA-qPCR methodology gives more realistic results for bacterial inactivation as they are very close to culture & counting results if process determined or induced an alteration of the cell wall. The loss of viability of bacterial cells by thermal effect can be accounted by a loss of integrity of many components of the bacterium, including the cell wall constituents. Therefore, a methodology like EMA-based qPCR will be able to penetrate thermal injured cells so that a positive EMA signal is observed for either thermal injured cells or wall injured cells by other ways. For that reason, the results of viable bacteria detected by EMA-qPCR are very similar to non-viable or non-culturable (culture & count method) results, opposite to qPCR results where cell integrity cannot be detected. Similar coherence between cuture&count and EMA-qPCR

results will be expected for other bacterial inactivation processes that induce cell wall injure.

3.3 Solar and H_2O_2/solar inactivation of L. jordanis in distilled water

Figure 3 shows the inactivation of *L. jordanis* under natural sunlight in distilled water by H_2O_2/solar at low concentrations, 10, 20 and 50 mg/L of H_2O_2 and solar disinfection. Prior to solar test, toxicity of each H_2O_2 concentration herein was evaluated in the dark. Results demonstrated non-toxic effects on the viability of bacteria as initial *L. jordanis* concentration, in terms of CFU/mL and CE/mL, measured by culture&counting technique and EMA-qPCR, remained constant over 5 h at each H_2O_2 concentration investigated (data not shown).

Solar only disinfection results showed that complete removal of bacteria from 10^6 CFU/mL to DL was achieved in 1.5 h of solar exposure. On the other hand, concentration of bacteria determined by EMA-qPCR showed a very different behavior, as no reduction on the concentration (CE/mL) was observed at all.

According to based-culture technique, the addition of low concentrations of H_2O_2 enhanced drastically the inactivation results compared to the use of solar only disinfection, as it has been previously shown [18, 20, 23]. The higher H_2O_2 concentration (50 mg/L), the higher inactivation kinetics was observed, requiring only 15 min~~utes~~ to reach the DL. Meaningfully, EMA-qPCR results reveal that ~~CE concentration was not decreased by this treatment at any H_2O_2 concentration, as if~~ no inactivation of *L. jordanis* cells occurred during the solar/H_2O_2 exposure, as bacterial concentration detected remained constant during all the experimental time and at any H_2O_2 concentration. Nevertheless, these results can be explained by the mode of functioning of the EMA-qPCR discrimination protocol that distinguishes between alive and dead cells. Briefly, EMA molecule diffuses inside cells with compromised membrane, where it covalently binds with DNA [9]. Consequently, this modification inhibits PCR amplification of DNA for dead cells or better 'membrane injured cells', allowing selective PCR amplification of unmodified DNA only for alive cells [9]. Regarding the bacterial inactivation mechanisms of these solar treatments, it is recognized that inactivation by H_2O_2/sunlight is basically originated by the generation of internal Reactive Oxygen Species (ROS) which produce continuous accumulation of

293 damages, oxidative reactions with intracellular components which eventually led to cell
294 death [18, 23]. At view of these results, we are confirming this mechanism with new
295 experimental evidence, and suggesting that the integrity of cell membrane is not altered
296 under this process, as diffusion of EMA was inhibited. Therefore, in the case of
297 H_2O_2/solar inactivation process, the EMA-qPCR technique couldn't permit to detect and
298 quantify injured cells as its mechanism of action doesn't alter the integrity of cells'
299 membrane, while culture-based quantification method identified a significant loss of the
300 number of viable *Legionella* cells.
301
302 ***3.4 Solar photocatalytic (TiO_2) inactivation of L. jordanis in distilled water***
303 Fig. 4a shows the inactivation of *L. jordanis* in distilled water under solar light in the
304 presence of TiO_2 at concentrations 100, 200, 300, 400, and 500 mg/L quantified by
305 culture-based method. It can be observed that the higher catalyst concentration the faster
306 inactivation kinetics, with the fastest case at 500 mg/L, where 15 min of solar exposure
307 led to reach the DL. Same experiments were monitored with EMA-qPCR (Fig. 4b). A
308 similar reduction on the bacterial concentration during exposure that becomes faster
309 with increasing TiO_2 concentration was observed. As opposite to the previous results
310 with solar/H_2O_2, it is well described in literature that inactivation mechanisms of TiO_2
311 primarily occurs by external damages of the cell wall [24], which favours the diffusion
312 of EMA into cells and binding with DNA from death or compromise bacteria,
313 permitting a positive detection of damaged cells using the EMA-qPCR, which is
314 consistent with culture-based quantification results (Fig. 4a).
315
316 For the case of 100 and 200 mg/L of TiO_2, a 4-log decrease of culturable bacteria was
317 observed after 30 min, while the concentration of CE/mL at that time, determined by
318 EMA-qPCR, remained nearly constant. This result clearly shows that the loss of cell
319 membrane integrity will occur only when the oxidative attack is sufficiently strong, as
320 for 300, 400 and 500 mg/L of TiO_2, where CE/mL reached DL. Therefore, EMA-qPCR
321 method is underestimating the number of undamaged cells, so that when the DL is
322 reached by this technique, the guarantee of the absence of any viable cell is more certain
323 than with culture-based methods. This is in agreement with other contributions related
324 to molecular techniques used for environmental samples [25]. At view of these results,
325 it can be established a direct correlation between EMA-qPCR and culturable counting

for samples treated with TiO_2/solar only when enough damages over cell wall are generated, and in this treatment this occurs with 300, 400 and 500 mg/L of catalyst.

To our knowledge, there are few contributions on the use of molecular techniques for the evaluation of photocatalytic disinfection efficiency. Chatzisymeon et al., 2011 reported the effect of *E. coli* inactivation by UV-A and TiO_2 (range from 50 to 400 mg/L) using both culture-based and qPCR method for bacterial quantification. These authors reported the capability of qPCR technique for the detection and quantification of bacterial DNA in water and wastewater samples during TiO_2 disinfection [25]. They found that the higher catalyst load the higher inactivation rate was observed, although different inactivation times were found for both quantification techniques, which were attributed to the phenomenon of "viable but not culturable bacteria" [25]. We confirmed the same tendency with photocatalytic inactivation of *L. jordanis*. On the other hand, Venieri et al., 2013 investigated the presence of two genes carried in two plasmids pXO1 and pXO2, respectively to identify potential resistance mechanisms of *B. anthracis* in water against photocatalytic (UVA/TiO_2), photolytic (UVC) and sonochemical treatments using also PCR technique [26].

In addition, it can be noted that initial concentrations of bacteria (CE/mL) detected by EMA-qPCR was lower than the expected and it decreased as TiO_2 concentration raised, i.e., from 100 to 500 mg/L (Fig. 4b). This effect is not observed in the case of quantification by based-culture technique as in all cases initial concentration of *L. jordanis* were ~ $5x10^5$ CFU/mL (Fig. 4a). ~~Moreover, It is well known that TiO_2 is not toxic (even at the highest concentration of 500 mg/L) for bacteria [27].~~ Comparing initial concentrations it is observed a lower detection of cells in the case of EMA-qPCR of 1.6, 2.0, 2.2, 1.1, and 2.5-log from 100, 200, 300, 400 and 500 mg/L of TiO_2, respectively, compared to based-culture technique, where bacterial concentration was constant. This reduction in detected cells may be explained as follows, during DNA extraction procedure a percentage of bacteria may be aggregated with TiO_2 particles which were eliminated during the extraction protocol; however it cannot be established a direct correlation between concentration of TiO_2 and efficiency of DNA extraction, as from 100 to 500 mg/L, there is 5-fold more particles while reduction of the bacteria detection is only 1.5-fold lower with 500 mg/L than with 100 mg/L. Nevertheless, this gap in the initial concentration of CE/mL detected after DNA extraction protocol is

360 neither affecting the validity of the EMA-qPCR results nor the conclusions obtained
361 from them, as the presence of TiO_2 particles doesn't inhibit DNA amplification or EMA
362 signal. On the other hand, these results may be also explained by the possible increased
363 bacterial susceptibility in the presence of TiO_2. Although, it is well recognized that TiO_2
364 is not toxic (even at the highest concentration of 500 mg/L) for bacteria [27], Pigeot-
365 Remy et al., 2011 reported experimental evidences of membrane integrity modification
366 in *E. coli* cell when exposed to TiO_2 in dark [7]. Our EMA-qPCR results confirm also
367 the modification of membrane integrity as found by these authors.
368
369 ### 3.5 Solar photocatalytic (TiO_2-H_2O_2) inactivation of *L. jordanis* in distilled water
370 Figure 5a shows the inactivation results of *L. jordanis* using TiO_2-H_2O_2 under sunlight
371 at concentrations of 100-10 mg/L, 200-10 mg/L and 500-10 mg/L. These results showed
372 the highest inactivation kinetics according to culture-based technique as compared to
373 previous results. In this case, the disinfection process is a simultaneous action of TiO_2-
374 photocatalysis and H_2O_2 photoactivated by sunlight. TiO_2 will attack cell membrane
375 progressively, and at the same time H_2O_2/UVA radiation will drastically accelerate the
376 bacterial inactivation via internal photochemical processes, and also H_2O_2 is acting as
377 electron acceptor accelerating photocatalytic rate. Low concentration of H_2O_2 was used
378 herein because it is well established that an excess of H_2O_2 in this process may inhibit
379 or reduce the photocatalytic activity [28] due to its reaction with TiO_2 surface holes
380 [29].
381 As demonstrated in previous section, TiO_2-photocatalytic oxidative attack will
382 eventually provoke loses in cells' wall integrity, and this will be shown by EMA-qPCR
383 results. On the other hand, the accelerated effect of H_2O_2 will not provoke significant
384 cell wall damages (Fig. 3), and for that reason we observe a very fast decrease in
385 cultured cells (Fig. 5a) while it is slower for the measurement by EMA-qPCR (Fig. 5b).
386 Therefore, the enhancement in the inactivation performance of heterogeneous
387 photocatalysis by H_2O_2 was not assessed by EMA-qPCR quantification results, in
388 agreement with our previous results on H_2O_2/Solar inactivation.
389
390 ## CONCLUSIONS
391

392 The inactivation of *L. jordanis* in water by TiO_2/solar UVA, H_2O_2/solar and TiO_2/
393 H_2O_2/solar-UVA processes has been demonstrated in distilled water under natural
394 sunlight.
395
396 According to culture-based method, faster bacterial inactivation was obtained using
397 500-10 mg/L of TiO_2 and H_2O_2, respectively. Efficiency order of inactivation was:
398 TiO_2/H_2O_2/solar (5 min.) > TiO_2/solar (15 min.) ≈ H_2O_2/solar (15 min.) > Solar only
399 disinfection (90 min.).
400
401 EMA-qPCR results demonstrated to be a good method to detect damaged and dead cells
402 when the treatment affects the integrity of the cell's membrane. Inclusion of EMA
403 treatment in qPCR analysis resulted in substantial or complete reduction of false
404 positives.
405
406 However, in solar disinfection and H_2O_2/solar (at non-toxic concentrations), where
407 membrane integrity remain unaffected, this tool couldn´t discriminate between live and
408 dead cells, as the mechanism of action of H_2O_2/solar is attributed to internal oxidative
409 injures which doesn't alter the external membrane.
410
411 The EMA-qPCR method has been used as a lab tool for finding insights on the
412 mechanisms of action of H_2O_2/solar and TiO_2/solar for bacterial inactivation. This work
413 has corroborated the well accepted mechanism of TiO_2-photocatalysis via oxidative
414 attacks of the external cell membrane. As well, the proposed mechanism for H_2O_2/solar
415 based on internal photochemical reactions has been reinforced by the experimental
416 evidences of this article, as no damage of cell membrane was detected.
417

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479 **FIGURES AND TABLES CAPTIONS**

480

481 **Table 1.** Summary of standard curves for qPCR and EMA-qPCR: Amplification
482 efficiency (C_T value), detection limit and relationship with CE/mL.

483

484 **Figure 1**. Standard curves of the inverse relationship between cycle threshold (C_T) and
485 *L. jordanis* (CE/mL) for qPCR and EMA-qPCR.

486

487 **Figure 2**. *L. jordanis* viability measured by culture technique (-■-), qPCR (-▲-) and
488 EMA-qPCR (-●-) during dark exposure at constant temperature of (a) 45ºC, (b) 50ºC
489 and (c) 60ºC.

490

491 **Figure 3**. *L. jordanis* inactivation by H_2O_2/solar in distilled water with 10 (-■-), 20 (-
492 ▲-), and 50 mg/L (-●-) of H_2O_2 and solar disinfection (-♦-) using culture-based
493 technique (full symbols) and EMA-qPCR (open symbols).

494

495 **Figure 4.** *L. jordanis* inactivation by TiO_2/solar in distilled water at concentration of
496 100 mg/L (-■-), 200 mg/L (-●-), 300 mg/L (-▼-), 400 mg/L (-♦-) and 500 mg/L (-▲-)
497 of catalyst measured using (a) culture technique and (b) EMA-qPCR.

498

499 **Figure 5.** *L. jordanis* inactivation by TiO_2/H_2O_2/solar in distilled water at concentration
500 of 100-10 mg/L (-■-), 200-10 mg/L (-●-) and 500-10 mg/L (-▼-) of catalyst and
501 hydrogen peroxide respectively, measured using (a) culture technique and (b) EMA-
502 qPCR.

503

504 **TABLE 1**

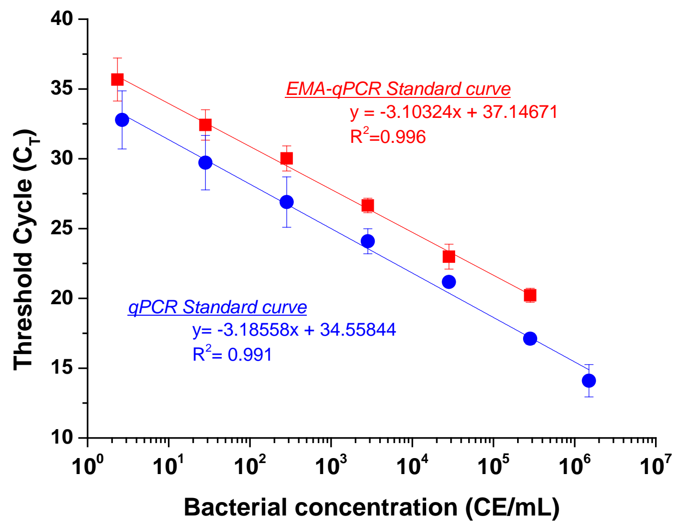
505

10-fold dilution of *L. jordanis* inoculum (CFU/mL)	C_T qPCR	C_T EMA-qPCR	CE/mL corresponding to each dilution
10^6	14.1019 ± 0.0001	ND	1500000 ± 900000
10^5	17.11 ± 1.16	20.2171 ± 0.0001	280000 ± 200000
10^4	21.2 ± 0.4	23.0 ± 0.9	28000 ± 20000
10^3	24.1 ± 0.3	26.7 ± 0.5	2800 ± 2000
10^2	26.9 ± 0.9	30.0 ± 0.9	280 ± 200
10^1	29.7 ± 1.8	32.4 ± 1.1	28 ± 20
10^0	32.9 ± 1.9	35.7 ± 1.5	3.0 ± 2.0
Negative control	No detected	No detected	
[a] Efficiency (%)	98.36	130.95	

506 [a] Average of the efficiency automatically calculated from Applied Biosystem 7500 Fast version v2.0.6 software

507

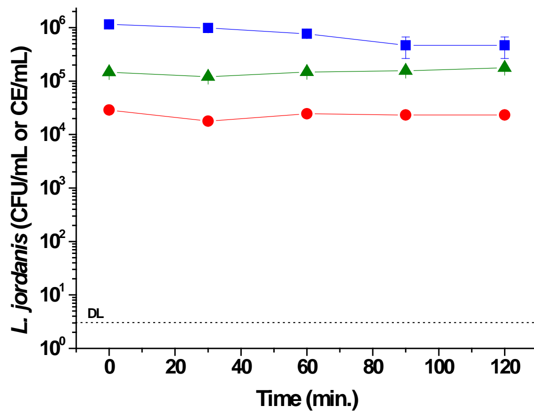
508 **FIGURE 1**



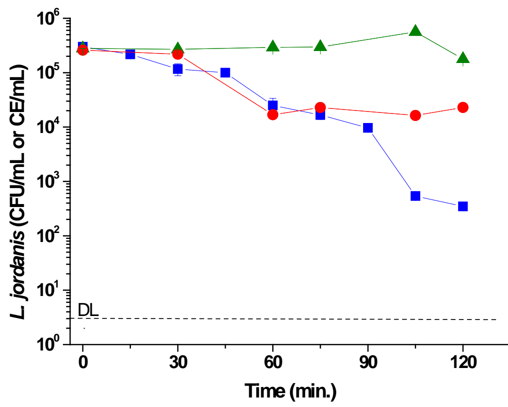
509

510

511 **FIGURE 2**



512 a)



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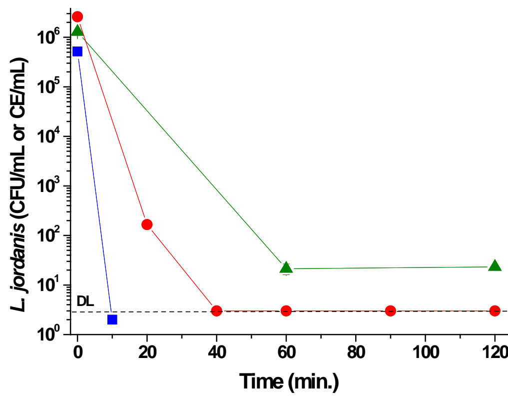
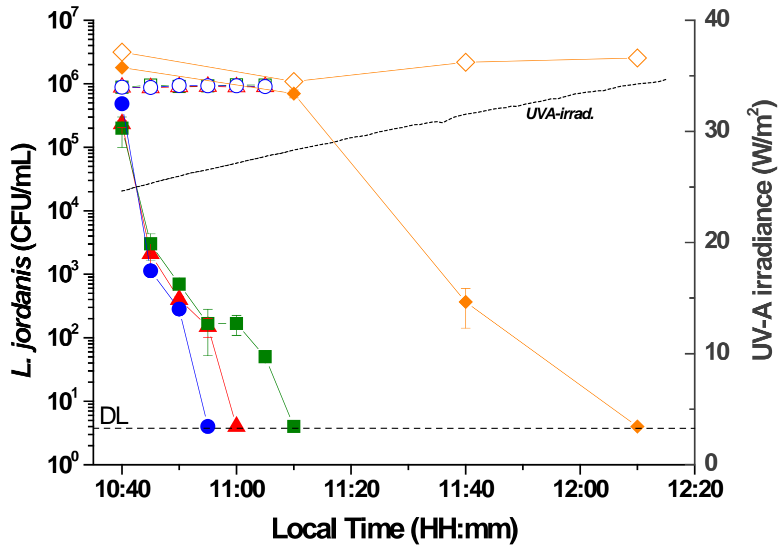


514 c)

515

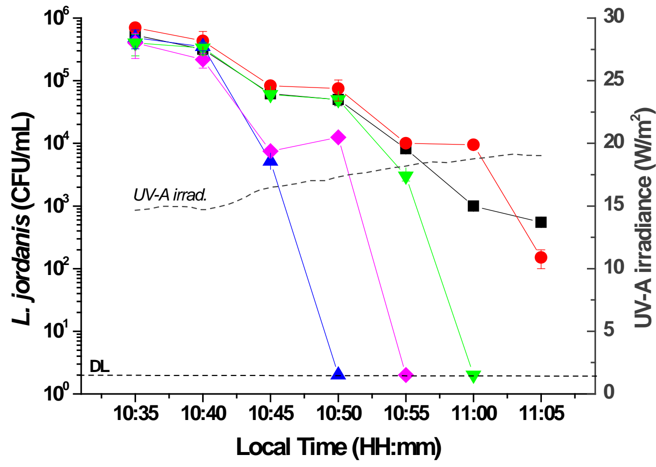
516 **FIGURE 3**



517

518

519 **FIGURE 4**



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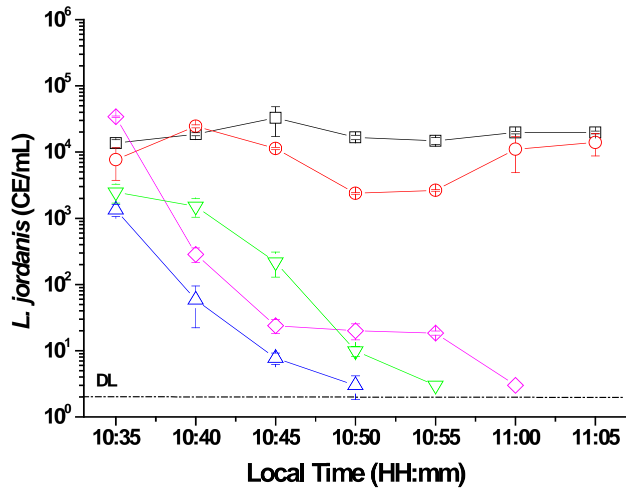
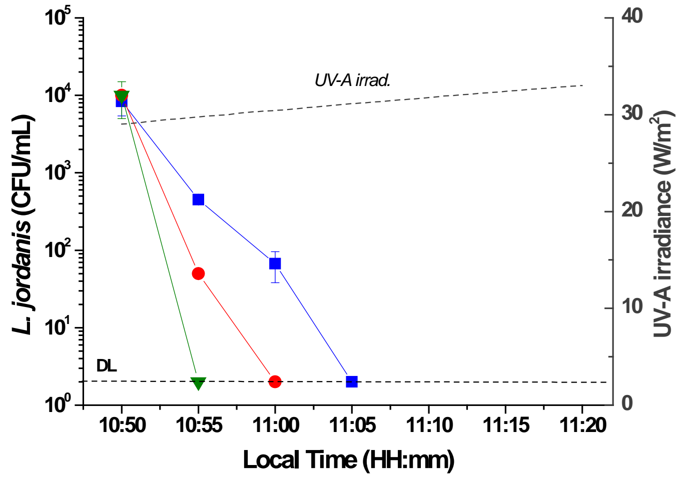
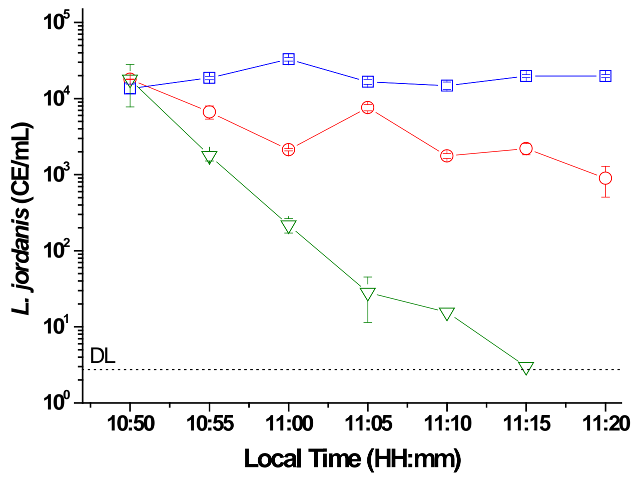


521 b)

522 **FIGURE 5**



523 a)



524 b)