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Inactivation of *Acanthamoeba* and its endosymbiont bacteria by the combination of solar light with H_2O_2

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ABSTRACT

Disinfection treatments are necessary for the safe use of water, but Free-Living Amoebae (FLA) are known to resist conventional processes, posing a health threat to water users that increases due to the endosymbiont bacteria (EB) FLA can carry inside. Advanced Oxidation Processes are promising disinfection treatments that have been extensively studied on bacteria but have barely been studied on FLA or EB yet. For the first time, the inactivation efficiency of combining H_2O_2 and simulated solar radiation (SR) against *Acanthamoeba* and their EB was evaluated. Its performance was compared to only H_2O_2 and solar radiation (SR) at 280–800 nm. The influence of *Acanthamoeba* origin was also evaluated. Concentrations of 7 to 10 mM H_2O_2 were necessary for inactivating *Acanthamoebae*, but 25 mM was required to kill EB. SR was inefficient for the used-to-solar-exposure *Acanthamoeba* strain. H_2O_2/SR improved the disinfection efficiency of treatments alone. 5 mM of H_2O_2/SR for 5 min eradicated both *Acanthamoebae* and EB, reducing up to 6 and 30 times the H_2O_2 dose and the SR fluence necessary, respectively. The *Acanthamoeba* strain that had already overcome water treatments was more resistant to all the treatments than the freshwater strain. This study underlines the protective role of amoebae in disinfection processes and the wide pathogenic microorganism spectrum that can overcome water treatments thanks to this "trojan horse". More research is needed to optimize conditions and establish H_2O_2/SR as an efficient disinfection treatment that prevents waterborne and nosocomial infections of endosymbiont microorganisms according to water use.

1. Introduction

Water disinfection is necessary to prevent waterborne diseases [1]. However, there are potentially pathogenic microorganisms that can overcome conventional water treatments and pose a public health threat [2]. Among them, free-living amoebae (FLA) are opportunistic pathogens that develop a cystic stage, which is very resistant to harsh conditions and disinfection treatments such as chlorine or ultraviolet radiation [3]. In addition, FLA can also become a reservoir of potentially pathogenic microorganisms since some bacteria -and other microbes- can survive inside them after being ingested by the amoeba or after infecting the amoeba. *Legionella* spp., *Mycobacterium* spp. or *Pseudomonas* spp., among others, are common endosymbiont bacteria transported by FLA [4]. Some species, such as *Vibrio cholerae* and *Legionella pneumophila* can even multiply inside FLA. In addition, endosymbiont bacteria can become more virulent and antibiotic-resistant after their intra-amoeba life [5].

Thanks to FLA protection, pathogenic endosymbiont bacteria can overcome water treatments [6], go through water systems unnoticed by microbiological controls, and colonize tap water, swimming pools, air conditioning devices, cooling towers or even hospital water systems, enhancing the risk of infection among water users [7]. As examples, Fernandez [8] reported the presence of potentially pathogenic endosymbiont bacteria inside FLA isolated from swimming pools and Muchesa et al. [9] detected amoeba-associated *L. pneumophila* in hospital water networks. Regulations play a key role in protecting people from possible infections and outbreaks; however, FLA are neglected across regulations related to water uses where FLA or endosymbiont

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infection results concerning [7]. For example, Greek and Italian regulations allow water reuse for cooling towers and condensers, but do not include any protozoa as a microbiological indicator control, underestimating the health risk [10]. These devices are typically colonized by FLA and become infective hotspots for endosymbiont bacteria such as *Legionella pneumophila* [5]. Thus, all these scenarios become a risk for public health and threaten the achievement of sustainable development goal number six, "water safety", from the 2030 Agency of United Nations [11]. To face this issue, disinfection treatments against FLA and the endosymbiont bacteria they protect inside must be evaluated.

Acanthamoeba spp. is the widest-spread and better-studied FLA genus. It is the most frequently found FLA genus, both in natural and artificial water [4,6], is the cause of different infections, such as keratitis or encephalitis, and can host a wide variety of potentially pathogenic bacteria inside [12]. So, it becomes an interesting model microorganism for studying FLA. Several studies reported that *Acanthamoeba* spp. can resist conventional disinfection processes such as chlorination, ultraviolet radiation or ozonization [13]. Thus, alternative disinfection treatments are necessary.

Hydrogen peroxide (H_2O_2) is known to be one of the most efficient *Acanthamoeba* disinfectants for contact lens care solutions [14]. This compound is also employed as a disinfectant for health-care settings, pipe disinfection and surface decontamination [15]. Indeed, this disinfectant was considered a control mechanism for *Legionella* in the water network of an Italian hospital [16]. However, as far as the authors know, H_2O_2 efficiency against endosymbiont bacteria protected by *Acanthamoeba* has never been evaluated.

Disinfection with UV radiation has been gaining traction in water disinfection in recent years. Its germicidal effect lies in damaging the DNA of microorganisms, impairing their growth or replication [17]. It is an interesting treatment that avoids the formation of the carcinogenic organochlorides produced by chlorination [18]. The high energy input requirement of UV lamps is a disadvantage to consider, especially in developing areas. Thereby, solar radiation, as a natural UV source, is gaining interest as an environmentally sustainable and low-cost disinfection treatment [17]. However, its efficiency varies depending on the targeted microorganism. Lonnen et al. [19] reported that a 5-log reduction of *Escherichia coli* and *Pseudomonas aeruginosa* was achieved after 2.5 h of solar radiation, while *A. polyphaga* cysts showed less than a 1-log reduction after 8 h of treatment. Thus, protozoa and, probably more worrisome, endosymbiont bacteria are still an open issue in solar radiation disinfection.

The inactivation of microorganisms can be improved by Advanced Oxidation Processes (AOPs). AOPs rely on hydroxyl radicals (˙OH) and other reactive oxygen species (ROS) formation, very reactive molecules that attack non-selectively, degrading compounds and inactivating microorganisms [20]. Solar-driven AOPs, also found in literature as photo-induced AOPs, have raised interest in the last decades as long as they reduce considerably treatment costs and turn the treatment into a clean technology [21,22]. Combining H_2O_2 and solar radiation (H_2O_2/SR) turns into a solar-driven AOP, known to be effective as a disinfection treatment against *Escherichia coli* [23], total faecal coliforms, *Enterococcus* [22], *Legionella jordanis* [24], *Pseudomonas aeruginosa,* MS2 virus [25], somatic coliphages, F-specific RNA bacteriophages [26], *Fusarium solani* [27] and *Fusarium equiseti* chlamydospores [28]. H_2O_2/SR avoids photocatalyst removal or pH corrections post-treatments as long as H_2O_2 auto-decomposes into water and oxygen [29], turning the treatment into an advantageous solar-driven AOPs. Indeed, it has been evaluated as a wastewater treatment for small urban wastewater treatment plants [23], as a tertiary wastewater treatment [30] and for reclamation purposes [26]. Thinking of *Acanthamoeba* and BE public health risks, H_2O_2/SR seems like an attractive process for disinfecting closed-water systems where these microorganisms can be specially threatening, such as swimming-pool, spas, hospital or health-care water systems.

The efficiency of some AOPs and solar-driven AOPs, such as chlorine photolysis or TiO_2 photocatalysis, against amoebae [19,31] and, in a few cases, against bacteria protected by amoebae [32,33] has already been evaluated, but the efficiency of H_2O_2/SR has never been evaluated against *Acanthamoeba* or their endosymbiont bacteria. Thus, the main goal of the present study is to evaluate the efficiency of combining H_2O_2 and simulated solar radiation (H_2O_2/SR) inactivating *Acanthamoeba* (FLA) and their endosymbiont bacteria (EB) protected inside the isolated amoeba. The efficiency of H_2O_2/SR was compared to the efficiency of H_2O_2 and solar radiation (280–800 nm) treatments alone. The influence of H_2O_2 and solar radiation (SR) doses and the water source where *Acanthamoeba* strains come from were also evaluated.

2. Materials and methods

2.1. *Acanthamoeba* strains evaluated

Two different FLA strains were tested: *Acanthamoeba* P31 (GenBank accession No. KY038362), isolated from an open swimming pool in Zaragoza, Spain [34] and *Acanthamoeba* C1–211, isolated from the freshwater of the river Noguera Ribagorzana in Lérida, Spain. Isolation was performed as described by Garcia et al. [4], and PCR and further sequencing were performed as described by Schroeder et al. [35]. The sequence was compared with the GeneBank database with the BLAST bioinformatic tool, and it was registered under the accession number OQ927217.

Both strains were grown at 30 °C in protease peptone, yeast extract and glucose (PPYG) medium supplemented with 250 μg/mL streptomycin, 250 μg/mL penicillin and 30 μg/mL fungizone to avoid contamination until trophozoite encystation occurred.

2.2. Disinfection treatments: preparation, conditions, development and sampling

Disinfection treatments evaluated were hydrogen peroxide (H_2O_2) and its combination with solar radiation (H_2O_2/SR). Before each treatment, FLA inoculums were prepared by centrifugation at 6000*g* for 10 min from PPYG medium axenic cultures and washing in a saline solution (0.9% NaCl) afterward. Taking a 3-log removal as a criterion of efficient disinfection [36], the FLA initial concentration was adjusted between $1 \cdot 10^4$ to $2 \cdot 10^4$ cells/mL. All the assays were developed at room temperature in batch reactors with a perfect blend. Reactors consisted of sterilized pyrex glass open beakers with a volume of 20 mL under constant agitation. H_2O_2 disinfection assays were developed in dark conditions and prepared by diluting hydrogen peroxide 30%, v/v, Panreac Química S.L.U. (Barcelona, Spain) in ultrapure sterilized water. H_2O_2/SR assays were conducted in an Atlas Suntest CPS+ /XLS+ solar chamber provided with a xenon lamp. This system reproduces natural sunlight conditions. Samples were exposed to wavelengths between 280 and 800 nm. All the essays were carried out with 500 W/m^2 which corresponds to 50% of the light intensity of the midday equatorial solar radiation [17]. The temperature was maintained below 30 ºC during the assays so that only solar radiation (and not heating) was involved during the disinfection process.

Concentrations of H_2O_2 studied ranged between 1 and 25 mM (34 and 1700 mg/L), according to a previous screening test [37]. The maximum exposure time was set to 30 min. For H_2O_2 assays, aliquots were sampled at the initial and final times of the assay. In the case of H_2O_2/SR assays, samples were also taken during the experiment. An assay without H_2O_2 in darkness was evaluated as a control. To determine only the SR effect, an assay without H_2O_2 under SR was also evaluated. The survival of *Acanthamoeba* (FLA), the survival of their endosymbiont bacteria (EB), and the residual H_2O_2 concentration were evaluated from aliquots. Assays were performed in duplicate.

H_2O_2 initial and residual concentrations were measured following the spectrophotometric method using metavanadate from Nogueira et al. [38]. Absorbance was read by a Jenway™ 6305 UV/visible

spectrophotometer (Fisher Scientific S.L., Madrid, Spain) at 450 nm.

SR exposure was quantified by the SR dose or fluence (F), which is calculated as the product of the light intensity and the exposure time: F = light intensity (W/m^2) × time (s), in W.s/m^2 [39,40].

2.3. Determination of *Acanthamoeba* inactivation

The survival of *Acanthamoeba* was determined using the most probable number (MPN) procedure adapted from Beattie et al. [41]. A sodium thiosulfate solution was added to the aliquots for H_2O_2 neutralization. 10 μL of the aliquots sampled and their dilutions (10^0, 10^{-1}, 10^{-2}) were inoculated onto Non-Nutrient Agar (NNA) plates covered by heat-inactivated *Escherichia coli.* Each inoculation was performed in quintuplicate. Also, 100 μL of the aliquots sampled were inoculated in quintuplicate on a plate. Plates were incubated during 15 days at 30 °C and regularly observed under an optical microscope looking for presence or absence of growth. Results were reported using an MPN table [41]. If no amoebic growth was detected in the 10 μL aliquots, then the 100 μL aliquots were checked for growth; no growth indicated total kill of the amoebae.

FLA inactivation was defined as a logarithmic reduction (N/N_0), where N_0 and N were the most probable numbers of viable cells before and after *t* time of the treatments. For H_2O_2 assays, FLA inactivation was recorded as a function of H_2O_2 concentration (mmol/L and mg/L). In the case of H_2O_2/SR assays, FLA inactivation was recorded as a function of exposure time (min) and as a function of fluence (KW.s/m^2).

The necessary H_2O_2 concentration and fluence for a 2-log ($[H_2O_2]_{99\%}$, $F_{99\%}$) and a 3-log ($[H_2O_2]_{99.9\%}$, $F_{99.9\%}$) FLA reduction were estimated from the linear regression section of the FLA inactivation curves. FLA inactivation curves were obtained by Microsoft Excel 365 Software.

2.4. Determination of EB inactivation

The survival of the environmental EB protected and carried by the *Acanthamoeba* under study was determined qualitatively. A sodium thiosulfate solution was added to the aliquots for H_2O_2 neutralization. 100 μL of the aliquots sampled were inoculated in quintuplicate on a Müeller Hinton plate. Plates were incubated during 48 h at 37 °C and observed for the presence or absence of growth. Aliquots sampled from control assays were used to compare endosymbiont bacterial growth. Results are expressed as three qualitative categories: non-affected survival if bacteria growth was similar to control; affected survival if a reduction of bacteria growth was appreciated compared to control; and inactivation of bacteria if no bacteria growth was detected after the treatment. The necessary H_2O_2 concentration (mmol/L) and F (KW.s/m^2) for EB total inactivation were described by the conditions that led to a total absence of bacterial growth under the conditions studied.

3. Results and discussion

3.1. Inactivation of *Acanthamoeba* by H_2O_2 treatment

H_2O_2 has been used for many years as a disinfectant for drinking water treatments, decontamination of medical devices [42] or biofilm growth control [43] due to its efficacy against a wide spectrum of microorganisms and reasonable manipulation safety. It is also considered an environmentally friendly treatment as long as no toxic by-products are produced, in contrast to chlorination treatments [16]. The efficiency of H_2O_2 against *Acanthamoeba* has been especially studied for contact lens formulation purposes [13] but, as far as the authors know, never for water treatment purposes, which are less demanding.

To determine the disinfection effectiveness of H_2O_2, *Acanthamoeba* inactivation was evaluated after 30 min of exposure to H_2O_2 concentrations that varied from 1 to 25 mM (Fig. 1). As H_2O_2 concentration increased, the number of *Acanthamoeba* cells was gradually reduced.

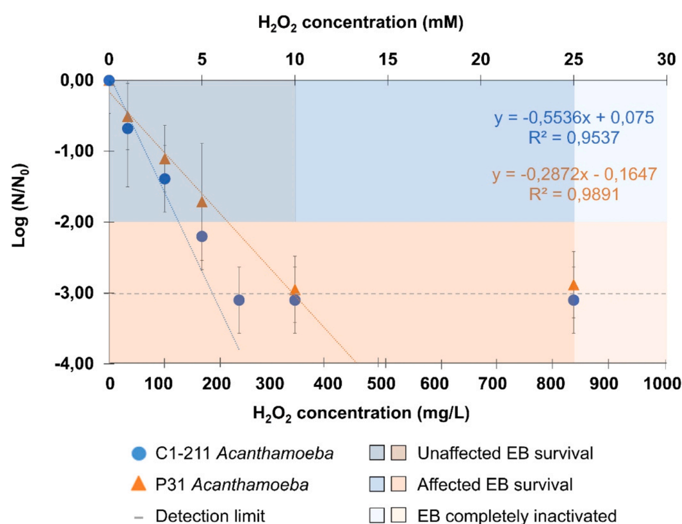

Fig. 1. Inactivation of *Acanthamoeba* and their EB after H_2O_2 treatment. The darkest blue (C1–211) and orange (P31) back colors indicate that EB survival was not affected; the intermediate blue and orange colors indicate that EB survival was affected; the clearest blue and orange colors indicate that EB were totally inactivated.

Total inactivation was reached after exposure to 7 mM (238 mg/L) and 10 mM (340 mg/L) H_2O_2 for 30 min in the cases of C1–211 and P31 *Acanthamoeba*, respectively. Necessary H_2O_2 doses for achieving a 2-log and 3-log reduction of *Acanthamoebae* (Table 1) were estimated through the equation obtained from the linear part of the graphic (Fig. 1). C1–211 *Acanthamoeba* required 3.7 and 5.6 mM of H_2O_2 to be reduced 2 and 3 logarithmic units, respectively. P31 *Acanthamoeba* required higher doses: 6.4 and 9.9 for a 2- and a 3-log reduction, respectively.

Comparing with the literature, the evaluated strains were much more sensitive than most of the *Acanthamoeba* strains evaluated so far [13], but most of these studies correspond to contact lens formulation analyses in which high doses (in the molar range rather than millimoles) and especially resistant *Acanthamoeba* strains are evaluated. The sensibility of FLA to disinfectants is highly variable among amoeba genera, species and strains; for example, Coulon et al. [3] reported resistances to H_2O_2 among different *Acanthamoeba* strains that varied from less than a 1-log reduction after 30 min to a 4.8-log reduction after 20 min. It might also be considered that such high resistances reported before corresponded to cysts, while the inoculums here evaluated contain some trophozoites and immature cysts, which are more sensitive than cysts [44].

The *Acanthamoeba* cyst wall is composed of two highly impermeable layers, the endocyst and the ectocyst, made up of a complex polysaccharide based on glycans and cellulose fibrils [45]. Pores are found where the endocyst and the ectocyst meet. The number of these pores, the cyst wall thickness and the cystic age might influence the susceptibility to chemical disinfectants and biocides of every strain [46]. This might explain the differences found in C1–211 and P31 *Acanthamoeba* H_2O_2 susceptibility, and also the differences with *Acanthamoeba* strains reported before, though other factors might also be involved. It should be considered that H_2O_2 disinfection studies are difficult to compare as long as experimental conditions are completely different regarding disinfection purpose, strains targeted, water matrix or contact time. Even units of measure lack standardization: mg/L, % (w/v), % (v/v) or mM are mixed across literature [15].

3.2. Inactivation of endosymbiont bacteria by H_2O_2 treatment

The bacterial vector and protector roles of amoebae have been widely studied so far. Several reviews reported the huge variety of

Table 1
Disinfectant doses for a 2-log and a 3-log *Acanthamoeba* reduction and EB eradication. H_2O_2 concentration (mM) and fluence (KW.s/m^2) for a 2-log ($[H_2O_2]_{99\%}$ and $F_{99\%}$) and 3-log ($[H_2O_2]_{99.9\%}$ and $F_{99.9\%}$) reduction of FLA and for eradication of EB ($[H_2O_2]_{EB}$ and F_{EB}).

	C1-211			P31		
Treatment	$[H_2O_2]_{99\%}$	$[H_2O_2]_{99.9\%}$	$[H_2O_2]_{EB}$	$[H_2O_2]_{99\%}$	$[H_2O_2]_{99.9\%}$	$[H_2O_2]_{EB}$
H_2O_2	3.7	5.6	25	6.4	9.9	25
Treatment	$F_{99\%}$	$F_{99.9\%}$	F_{EB}	$F_{99\%}$	$F_{99.9\%}$	F_{EB}
SR	162.1	292.3	900	-	-	> 900
1 mM H_2O_2/SR	57.3	87.4	900	163.3	277.2	900
3 mM H_2O_2/SR	47.2	68.6	300	34.7	56.7	900
5 mM H_2O_2/SR	10.6	15.8	150	19.1	29.0	150

bacteria genera -and other microorganisms- amoebae can carry inside, many of them potentially pathogenic for human health [5,7]. However, disinfecting bacteria protected inside amoebae has barely been studied; only chlorine, monochloramine, chlorine dioxide, UV$_{254\ nm}$ and chlorine photolysis against bacteria protected by amoebae have been evaluated so far [33,36,47]. Thus, this is the first study in which the effect of H_2O_2 disinfecting bacteria protected by amoebae was analyzed.

To determine the inactivation effectiveness of H_2O_2 against EB, bacteria's survival was evaluated by the presence or absence of bacterial growth after 30 min of exposure to H_2O_2 concentrations that varied from 1 to 25 mM. In Fig. 1, colors represent the three qualitative categories established for EB survival. Results show that both C1–211 and P31 EB required an exposure time of 30 min to 25 mM (850 mg/L) H_2O_2 to get inactivated. According to Mohammed [48], 35 mg/L of H_2O_2 (1 mM) can control waterborne bacteria, suggesting that bacteria might resist higher H_2O_2 doses when protected inside amoebae than free bacteria. This fact was already demonstrated with chlorine; Garcia et al. [49] reported that *L. pneumophila* resisted four times higher chlorine doses while being protected by *A. polyphaga.*

Comparing with H_2O_2 inactivating FLA results, between 2.5 and 3.5 higher disinfectant doses were necessary to inactivate EB than to inactivate the *Acanthamoeba* that carries them inside (Fig. 1 and Table 1). This agrees with reports by He et al. [47], who found that the *Burkholderia* bacterium required higher doses of chlorine and chlorine dioxide than its protective *Dictyostelium discoideum* amoeba to get inactivated. These results underline the strong protective role of amoebae, which might be related to the cyst wall's robustness and the H_2O_2 action mechanism.

H_2O_2 affects microorganisms through internal and external damage [43]. On the one hand, internal damage is related to ·OH radical generation through intracellular Fenton reactions, produced when H_2O_2 is combined with mainly ferrous but also ferric inner ions [50], DNA damage [43] and mitochondrial function affection [51]. On the other hand, the external mechanism of H_2O_2 damage is based on the cell wall attack and permeability increase, which allow extra H_2O_2 inflow and further damage as well as an overall detrimental effect on cell viability [43].

As shown in Fig. 1, P31 EB survival was affected by all the H_2O_2 doses evaluated. On the contrary, C1–211 EB survival remained unaffected until C1–211 *Acanthamoeba* was completely inactivated. A higher proportion of P31 trophozoites than cysts might be present during the assays, which are more sensitive, and the affected P31 EB at lower H_2O_2 doses might be those liberated by the affected and dead trophozoites. Regarding C1–211 results, its EB might be well protected and not released outside, even though the disinfectant might penetrate C1–211 trophozoites and cysts and inactivate them. Maybe the cell compartment endosymbiotic bacteria inhabit inside amoebae [5] or the expulsion of vesicles fulfilled with EB by amoebae, which can also provide protection [7], may influence EB inactivation to some extent.

European Union regulations establish 25 mg/L (0.7 mM) H_2O_2 as the maximum dose for drinking water disinfection and accept 5 mg/L as the residual concentration [52]. The United States Environmental Protection Agency [39] recommends a residual concentration of H_2O_2 between 25 mg/L (0.7 mM) and 50 mg/L (1.5 mM) in drinking water. Taking these regulations into account, C1–211 and P31 *Acanthamoebae* as well as their EB, can easily overcome water treatment plants based on this disinfecting process, colonize water systems as pipes, taps, fountains or hospital showers, among others [7] and pose a health threat to people exposed. Thus, effective alternatives to H_2O_2 disinfection are necessary to inactivate *Acanthamoeba* and EB.

3.3. Inactivation of Acanthamoeba and endosymbiont bacteria by SR treatment

Solar radiation (SR) has been widely used due to its germicidal power. The wavelength range that arrives at the Earth comprises 290 nm to 800 nm that is, UV-B and UV-A [17], similar to the wavelength range used in this study. This radiation inactivates microorganisms in a direct (DNA dimer formation and damage) and indirect (oxidative damage produced by the increase of intracellular ROS and photo-Fenton reactions) way [43]. This environmentally friendly and cost-effective disinfection process proved to be especially effective against some bacteria and viruses, but protozoa are known to be more resistant [39].

To determine the effectiveness of SR, FLA and EB inactivation were evaluated at different times during 30 min of exposure to 500 W/m^2 of simulated SR. Necessary SR fluence for achieving a 2-log and 3-log reduction of *Acanthamoebae* (Table 1) were estimated through the equation obtained from the linear part of the graphic (Fig. 2.a and 2.b). Results show that FLA (Fig. 2.a and 2.b) and EB inactivation (Fig. 3) were dependent on the UV dose: the longer the exposure, the higher the inactivation rate achieved. C1–211 *Acanthamoeba* required 10 min of SR to be totally inactivated (F = 300 KW.s/m^2). In the case of P31 (Fig. 2.b), SR achieved a 2-log *Acanthamoeba* reduction during the first 5 min and it was maintained up to 30 min, not achieving total inactivation. P31 *Acanthamoeba* initial reduction might correspond with trophozoite or immature cyst inactivation (that can be present in the initial inoculum of the assays), whereas cysts might remain viable and require more than 900 KW.s/m^2 to be completely inactivated. This result agrees with reports by Lonnen et al. [19] who stated that SR$_{300\ nm\ -\ 10\ \mu m}$ was effective in inactivating *A. polyphaga* trophozoites, but ineffective against the cysts.

Comparisons with the literature are complicated due to differences in UV radiation wavelengths and light intensities. Still, C1–211 and P31 *Acanthamoeba* strains are more sensitive than the *A. castellanii* cysts irradiated with 550 W/m^2 of SR$_{290–800\ nm}$ that were 2-log reduced after 2 h of treatment ($F_{99\%}$ = 3960 KW.s/m^2) by Heaselgrave and Kilvington [53]. *Acanthamoeba* strains under study are also more sensitive than the *A. polyphaga* evaluated by Lonnen et al. [19], whose trophozoites achieved a 3-log reduction after 2 h ($F_{99.9\%}$ = 7200 KW.s/m^2), while cysts were reduced less than a 1-log after 8 h of 870 W/m^2 of SR$_{300\ nm\ -\ 10\ \mu m}$ (F = 28800 KW.s/m^2).

Like H_2O_2 disinfection assays, disinfectant doses to inactivate EB were higher than those necessary to inactivate their protective *Acanthamoeba*. C1–211 EB required three times the necessary fluence (F_{PB} = 900 KW.s/m^2) to totally inactivate the protective *Acanthamoeba*. In the

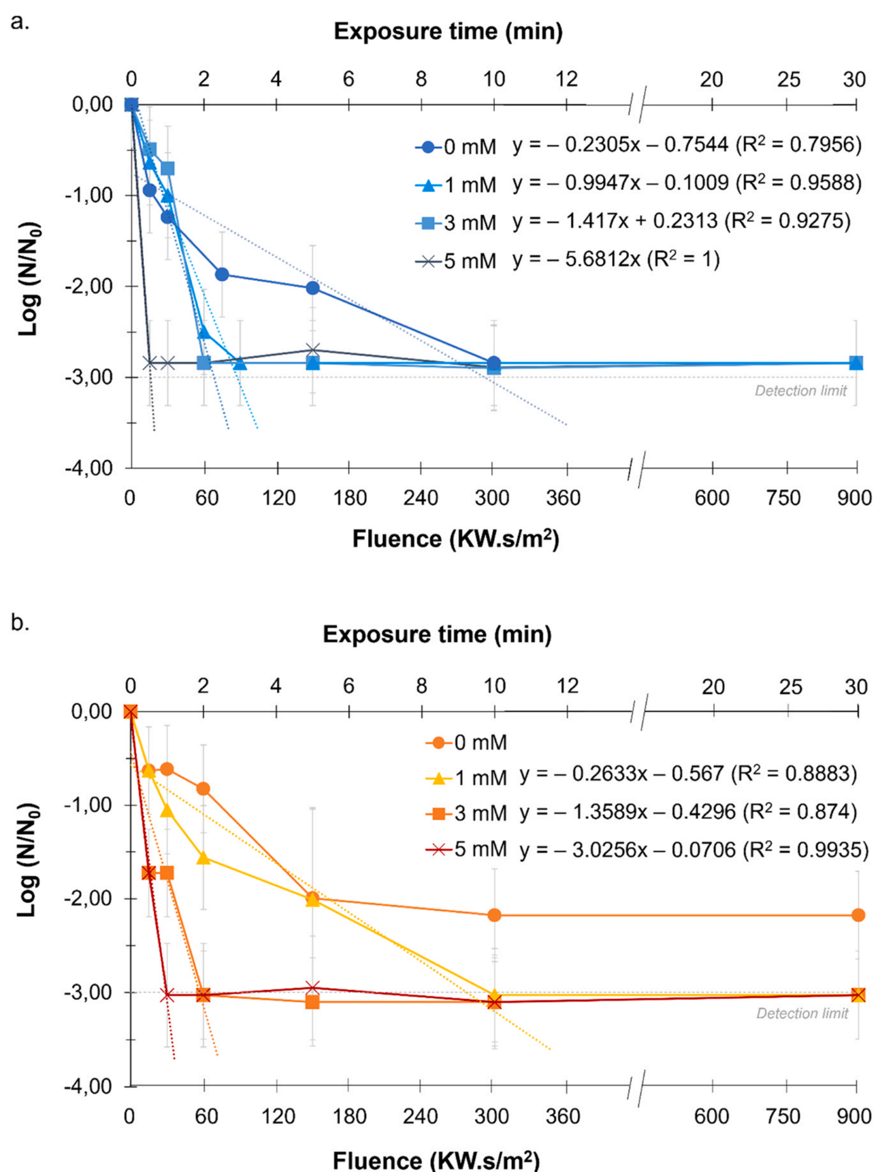

Fig. 2. Inactivation of (a) C1–211 and (b) P31 *Acanthamoeba* along SR and H_2O_2/SR treatments with different H_2O_2 doses.

case of P31 EB, since 30 min of SR exposure were not enough to inactivate P31 *Acanthamoeba*, they were also not enough to inactivate their EB (Fig. 3.b); thus, doses higher than 900 KW.s/m^2 of SR exposure are necessary. This fact was also reported by Adan et al. [32], who found that 150 min of UVA radiation barely affected *Escherichia coli* in the presence of *Acanthamoeba*. However, *E. coli* survival was not related to amoeba protection but to amoeba scattering of UV light. The present results also agree with the reports by He et al. [47], who described that *Dictyostelium discoideum* amoeba was able to protect the *Burkholderia* bacterium inside even after being inactivated by $UV_{254\ nm}$. He et al. [47] suggested that cysts can absorb and screen UV light, so that protected bacteria can only be attacked by a fraction of UV light.

As solar radiation did not achieve efficient inactivation of P31 *Acanthamoeba* or P31 EB, alternative disinfection treatments are still necessary.

3.4. Inactivation of Acanthamoeba and endosymbiont bacteria by H_2O_2/SR treatment

Combining H_2O_2 with solar radiation can become a highly efficient disinfection treatment that considers H_2O_2, solar radiation and Advanced Oxidation Process (AOP) advantages. The microbial inactivating mechanism of the H_2O_2/SR treatment is controversial and still remains unclear [26]. Most of the studies developed so far evaluate the inactivation of bacteria; however, this is the first study in which the inactivation of amoebae and endosymbiont bacteria has ever been evaluated.

To determine the effectiveness of H_2O_2/SR, *Acanthamoeba* (Fig. 2.a and 2.b) and EB (Fig. 3) inactivation were evaluated at different times during 30 min of exposure to H_2O_2 concentrations that varied from 1 to 25 mM combined with 500 W/m^2 of simulated SR. As a rule, results show that the H_2O_2 and SR combination enhanced the inactivation of both *Acanthamoeba* and EB, compared with H_2O_2 and SR treatments alone, which agrees with previous studies [22–28], in which the inactivation of *E. coli*, total faecal coliforms, *Enterococcus, Legionella jordanis, Pseudomonas aeruginosa*, MS2 virus, somatic coliphages, F-specific RNA bacteriophages *Fusarium solani* and *F. equiseti* by H_2O_2/SR was evaluated. Indeed, the total inactivation of P31 *Acanthamoeba* was achieved at any of the H_2O_2 doses evaluated under H_2O_2/SR experiments, while it was not achieved by only SR treatment. This agrees with reports by Chauque and Rott [31], who found that the NaOCl/UV AOP could inactivate *A. castellanii* while only UV radiation could not.

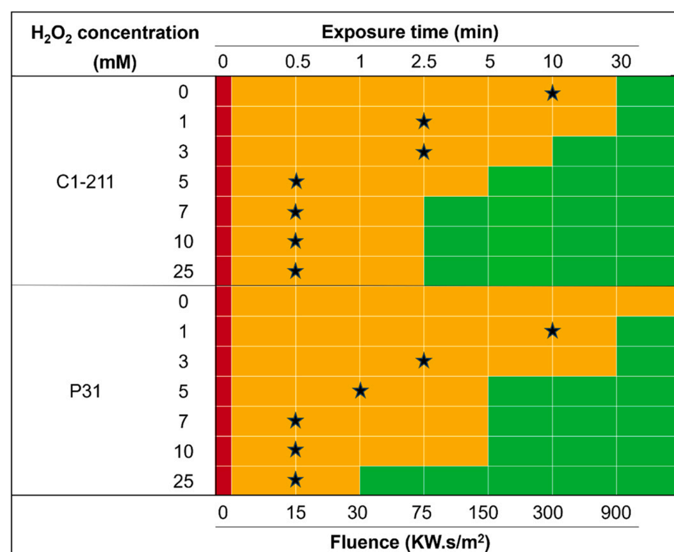

Fig. 3. Inactivation of C1–211 and P31 EB after SR and H_2O_2/SR treatments. Bacteria survival is semiquantitative indicated in colors: red color means unaffected bacterial survival, orange color means affected bacterial survival and green color means completely inactivated bacteria. Black stars indicate the exposure time and fluence necessary to inactivate the corresponding FLA at the different H_2O_2 doses evaluated.

Combining 1 mM H_2O_2 (34 mg/L) and SR, the necessary time and fluence to inactivate C1–211 and P31 FLA were reduced 3–4 folds compared to only SR treatment (from 300 and higher than 900 KW.s/m^2 to 75 and 300 KW.s/m^2, respectively), while EB inactivation was barely improved (F = 900 KW.s/m^2). 3 mM H_2O_2/SR sharpened the reduction of the necessary exposure time to inactivate *Acanthamoeba* P31 five times (60 W/m^2) but did not improve the EB inactivation (F_{EB} = 900 KW.s/m^2). In the case of C1–211, contrastingly, 3 mM H_2O_2/SR (102 mg/L) maintained FLA and reduced EB inactivation time and fluence to 300 KW.s/m^2. This fact was also observed in the 5 mM H_2O_2/SR (170 mg/L) assays against P31; FLA inactivation was barely improved compared to the 3 mM H_2O_2/SR treatment, while the necessary time and fluence to inactivate its EB were reduced. This suggests that, under 1 mM or 3 mM H_2O_2/SR treatment for C1–211 and P31, respectively, the inactivation mechanism(s) were not enough to reach and attack endosymbiont bacteria, but higher H_2O_2 doses might enhance them to a greater extent that causes EB inactivation.

Increasing H_2O_2 doses to 5 mM accelerated sharply both FLA and EB inactivation: C1–211 and P31 FLA were totally inactivated 20 and higher than 30 times faster (15 and 30 KW.s/m^2, respectively), and their EB were totally inactivated at least 6 times faster compared to only SR treatments (150 KW.s/m^2 for the EB of both *Acanthamoeba*). These results agree with reports by Polo-López et al. [24] and Martinez-Garcia et al. [25], who proved that 0.3 mM and 0.03 mM H_2O_2/SR reduced 3 and > 20 folds the necessary SR exposure time to inactivate *L. jordanis* and MS2 virus, and that increasing to 1.5 mM and 0.15 mM H_2O_2 enhanced inactivation until reducing 6 and 100 folds the necessary exposure time, respectively, compared with only SR treatment. Mamane et al. [20] evaluated the inactivation of different viruses and *E. coli* through H_2O_2/UV ($\lambda > 295$ nm) and also found that it increased with the initial H_2O_2 dose, agreeing with the results here obtained.

There is evidence that a synergistic effect between SR and H_2O_2 takes place [28]. The high efficiency of the H_2O_2/SR treatment might be due to the combination of multiple mechanisms that accumulate cellular damages and inactivate the FLA and EB under study: (1) the H_2O_2 internal and external damage, (2) the SR direct and indirect damage, (3) the damage produced by the ˙OH formed through H_2O_2 photolysis, though it might be lower than H_2O_2/UV-C treatments due to the H_2O_2 UV absorbance spectra [54]; and (4) the (photo-)Fenton reactions developed after inner iron ions release due to direct UV and indirect ROS damage to iron-containing clusters [43] and iron-containing proteins [28]. All these mechanisms produce highly reactive ROS and, especially, ˙OH, which is responsible for initiating free radical and cytotoxic reaction cascades that damage DNA, proteins and lipids. Temperature increase is also known to promote microbial inactivation during the H_2O_2/SR process [28], but under the experimental conditions stated, it is not considered for so, as long as it was controlled and maintained up to 30 °C throughout the treatments.

In addition, some reactions that take place intra- and extra-cellularly might feed back themselves; as shown in Table 2, in H_2O_2/SR treatments, H_2O_2 concentration showed an initial decrease but a latter increase that even implies concentrations higher than initial ones. Under solar light, H_2O_2 cellular regulation systems such as catalase and superoxide dismutase (SOD) enzymes, are hampered, leading to an H_2O_2 over-accumulation [55], which could be liberated once the amoebae and/or the bacteria are damaged, increasing extracellular levels.

Further and fundamental studies, as well as ultrastructural analyses similar to those developed by He et al. [47] and Wang et al. [33], should be developed to better understand the inactivation mechanisms that may occur during both *Acanthamoeba* and BE inactivation by H_2O_2/SR treatment and optimize disinfection conditions.

Increasing to 7 mM H_2O_2/SR reduced the necessary time to inactivate C1–211 EB to 2.5 min (75 W.s/m^2) and to 0.5 min (15 KW.s/m^2) the necessary time to inactivate P31 *Acanthamoeba*. 10 mM H_2O_2/SR did not improve *Acanthamoeba* or EB inactivation. 25 H_2O_2 mM combined with SR reduced the P31 EB eradicating time to 1 min (F_{EB} = 30 KW.s/m^2), while in the case of C1–211, results were maintained even though the H_2O_2 doses increased. The fact that the inactivation improvement with increasing H_2O_2 doses to 7 mM (238 mg/L) or higher is hardly significant might be due to a saturation effect of H_2O_2, while a decrease in the efficiency of degradation would be due to the H_2O_2 quencher effect [50]. Indeed, this saturation effect was also reported by Polo-Lopez al. [28] and Agulló-Barceló et al. [26], who reported that *Fusarium solani* and that *E. coli*, F-specific RNA bacteriophages, somatic coliphages and sulphite-reducing clostridia inactivation was the same at 15 and 10 mg/L, and at 20 and 50 mg/L, respectively. On the other hand, Adeel et al. [23] reported the quencher effect when evaluating *E. coli* inactivation through H_2O_2/SR: 40 mg/L (1.2 mM) of H_2O_2 improved the inactivation compared to SR, while 90 mg/L (2.6 mM) decreased it.

Considering the saturation effect and the results, 5 mM H_2O_2/SR (170 mg/L) during 5 min (F = 150 KW.s/m^2) stands as an effective disinfection treatment against both *Acanthamoebae* and EB. Lower H_2O_2 doses (between 1 and 50 mg/L, 0.03 and 1.5 mM) were reported to be efficient in H_2O_2/SR assays against bacteria and phages [23–26] and also against some fungal species [27,28], but were not efficient against more resistant microorganisms, such as *B. subtilis* spores [20], *Clostridium perfringens* spores [56] or *Cryptosporidium parvum* oocysts [57]. Thus, it would be interesting to evaluate the H_2O_2 doses here reported as efficient in further H_2O_2/SR studies against these resistant microorganisms.

3.5. Considerations, relevance of the study and future perspectives

P31 *Acanthamoeba* was more resistant than C1–211 to the three treatments compared (H_2O_2, SR and H_2O_2/SR). This might be related to differences in cystic wall composition, as commented in 3.1., but also to the water source. P31 *Acanthamoeba* was isolated from an outdoor swimming pool, where it is constantly exposed to solar radiation and chlorine. Hence, a higher resistance of P31 *Acanthamoeba* and its EB to the SR treatment can be expected. In addition, chlorine, when irradiated by solar radiation, produces ˙OH among other reactive species [31], and thus, this amoeba is used to the exposure and attack of ˙OH radicals. Therefore, P31 *Acanthamoeba* is understandably more resistant than

Table 2
H_2O_2 residual concentration (mM) in dark H_2O_2 and H_2O_2/SR experiments with diferent initial H_2O_2 doses.

Treatment	Time (min)	C1-211				P31			
		1 mM	3 mM	5 mM	10 mM	1 mM	3 mM	5 mM	10 mM
H_2O_2/SR	0	1.04	3.06	5.03	10.02	1.00	3.07	5.01	10.02
	2.5	0.99	2.96	4.85	9.74	1.54	1.70	4.92	8.79
	5	0.94	2.88	4.67	9.35	1.41	1.58	4.99	9.83
	10	0.92	2.93	4.31	10.30	1.03	1.42	5.03	9.94
	30	0.80	3.53	5.67	11.13	1.38	1.65	5.66	10.95
H_2O_2	30	0.99	2.77	4.60	9.88	0.68	2.09	3.56	9.80

C1–211 to the evaluated treatments.

Previous studies also reported that *Acanthamoeba* strains isolated from urban water, such as hospital water, were more resistant to H_2O_2 and other disinfectants than freshwater *Acanthamoeba* strains [3]. Water treatments select resistant strains that might get into water distribution systems. If ineffective disinfection treatments are included throughout the distribution systems, especially highly resistant *Acanthamoeba* strains might be selected. In addition, considering the increase in the reuse and reclaiming of water [10], *Acanthamoeba* and their endosymbiont bacteria might become recalcitrant in the urban water cycle. All this reinforces the necessity of introducing point-of-use treatments and revising regulated microbial indicators for determined water uses where *Acanthamoebae* and their potentially pathogenic endosymbiont bacteria could pose a health risk, such as swimming pools for elderly people or infants, hospital water or health-care settings. In this sense, point-of-use H_2O_2/SR disinfection could be an interesting alternative, but residual concentrations (Table 2) might be accordingly considered. Optimizations of doses and exposure times could lead to better-fitting treatments. Also, further H_2O_2/SR studies against different *Acanthamoeba* strains and FLA genera and against potential EB non-culturable by the techniques here evaluated are necessary so that optimal conditions for effective disinfection can be stated.

As a hypothesis, it is proposed that EB could be killed (a) once they release out of a dead and lysed *Acanthamoeba* trophozoite or immature cyst or (b) once the disinfectant (whether H_2O_2, ·OH and other ROS or SR) reaches the bacteria inside the *Acanthamoeba*. In the latter case, the efficiency of the disinfectant would depend (among others) on the location of the EB, as long as they can be distributed among different *Acanthamoeba* compartments (in vacuoles or between the walls of the endocyst and ectocyst, for example [5]).

FLA can carry a huge variety of potential pathogenic microorganisms inside; long lists of different bacteria, viruses including SARS-CoV-2 or fungi can be found across the literature [5,58]. Just like endosymbiont bacteria require higher doses than FLA to get completely inactivated, other potentially pathogenic organisms protected inside FLA might also require them. This sharpens the risk of recolonization of water systems and hampers pathogen control. In this sense, further research on disinfecting other microorganisms susceptible to being protected by FLA would be interesting to analyze so that a more complete vision of the protective and “trojan horse” roles of amoebae could be stated. Also, further studies considering different factors such as water matrix, turbidity, ions, quenchers or organic matter are needed.

4. CONCLUSIONS

The combination of H_2O_2 and simulated solar radiation (H_2O_2/SR) was evaluated to compare its effectiveness on the inactivation of two *Acanthamoeba* strains isolated from different water sources and their endosymbiont bacteria to H_2O_2 and SR treatments alone. H_2O_2 was sufficient to obtain eradication, whereas only SR was not. The disinfectant power of H_2O_2 and SR was greatly improved when combined. H_2O_2/SR was efficient in inactivating both *Acanthamoeba* and their EB, reducing the necessary H_2O_2 dose up to 5 times and the necessary SR exposure time up to 30 times.

EB required higher H_2O_2 doses and longer SR exposure times than their protective *Acanthamoeba* to be eradicated, indicating that EB inactivation needs first the *Acanthamoeba* inactivation, underlying the protective role of amoebae against disinfectants. The *Acanthamoeba* strain isolated from a swimming pool, where it is constantly exposed to chlorine and solar radiation, was more resistant than the freshwater strain to all the treatments evaluated, indicating that conventional disinfection treatments select resistant strains that will be pretty difficult to further remove. These strains can hence become recalcitrant in the urban water cycle and enable the possible recolonization of water devices by the potentially pathogenic bacteria carried inside.

Given the resistance of *Acanthamoeba* and their protective role, it is considered of interest to extend knowledge of the effectiveness of H_2O_2/SR on a wider spectrum of free-living amoebae and their endosymbiont microorganisms, including bacteria, viruses and fungi, in order to optimize disinfection conditions and develop H_2O_2/SR as an efficient treatment that could fit according to water uses, protecting users from potential waterborne and nosocomial infections.

Declaration of Competing Interest

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Data Availability

Data will be made available on request.

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