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Chlorine Photolysis: A Step Forward in Inactivating *Acanthamoeba* and Their Endosymbiont Bacteria

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Abstract: Chlorine and solar disinfection are widely used disinfectants in water treatment. However, certain potential pathogens can resist these methods, posing a public health risk. One such case is *Acanthamoeba*, a resistant free-living amoeba that protects pathogens inside from disinfection, thus endangering the health of water users. This work is the first evaluation of the inactivation efficiency achieved by combining NaClO (Cl_2) and solar radiation (SR) against two *Acanthamoeba* strains from different sources (freshwater and pool water) and their endosymbiont bacteria (EB). Amoebae were exposed to different Cl_2 doses (0–500 mg/L), SR wavelength ranges (280–800 nm and 320–800 nm), used as gold standards, and their combinations. The EB exhibited resistance to conventional Cl_2 and SR treatments, requiring up to 20 times higher disinfectant doses than those needed to inactivate their protective *Acanthamoeba*. The pool strain and its EB demonstrated greater resistance to all treatments compared to the freshwater strain. Treatments with Cl_2 (5 mg/L)/$SR_{280–800nm}$ completely inactivated both *Acanthamoeba* and EB of the freshwater strain, reducing up to 100 times the necessary Cl_2 doses, suggesting that chlorine photolysis is an attractive treatment for disinfecting freshwater and preventing waterborne diseases associated with *Acanthamoebae* and its EB.

Keywords: disinfection; UV chlorine photolysis; Advanced Oxidation Process; solar radiation; *Acanthamoeba*; endosymbiont bacteria

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1. Introduction

Safe water is necessary for public health, regardless of its use. Inadequate management can lead to water contamination, further impacting the health of millions of people [1]. For this reason, different disinfection methods have been developed as necessary processes to ensure safety in different water and reclaimed water uses [2]. Regulations also play a crucial role in ensuring safety by establishing microbiological and physicochemical requirements that water must meet to be suitable for its different usages. However, there are potentially pathogenic microorganisms capable of surviving disinfection treatments that are not routinely monitored [3]. That is the case with some Free-Living Amoebae (FLA).

FLA are ubiquitous protozoa that can act as opportunistic pathogens. They can cause, among others, amoebic keratitis, encephalitis, or even fatal meningoencephalitis, observed with *Naegleria fowleri* [4]. FLA primarily feed on bacteria, but some bacteria can survive within FLA, such as *Legionella* spp., *Mycobacterium* spp., or *Pseudomonas* spp. [5], becoming endosymbionts. Some species, such as *Vibrio cholerae* or *L. pneumophila*, can multiply within FLA [6], transforming them into potential reservoirs for pathogenic bacteria. Indeed, FLA are commonly known as "trojan horses" [7].

Usually, FLA undergo two different stages: the trophozoite, which is the metabolically active stage, and the cyst, the resistance stage. The encystation process involves the production of a robust, thick wall that confers huge resistance to the amoebae against harsh environmental conditions and disinfectants [8], enabling them to survive water treatments. Consequently, they have been frequently isolated from the effluents of wastewater treatment plants [9,10] and drinking water treatment plants [11]. Given their resistance to treatments, FLA and, more concerning, potentially pathogenic endosymbiont bacteria protected inside them can spread in reclaimed water [10], drinking water systems, swimming pools [12], hospital water systems [13], or fountains [14]. In these environments, endosymbiont bacteria can be released outside FLA and recolonize these locations, turning them into infective foci. Indeed, the swift recolonization of cooling, domestic, or hospital water systems by *Legionella* is suggested to be facilitated by FLA protection [13,15].

Efforts have been undertaken to develop water treatments capable of inactivating amoebae. However, most studies have concluded that conventional treatments are ineffective in inactivating amoebae, at least at the typical doses, established according to bacteria elimination criteria [16,17]. For example, chlorine, the most common disinfectant, is typically used in water processes at doses ranging from 1 to 5 mg/L. Nevertheless, some *Acanthamoeba* strains can survive concentrations exceeding 100 mg/L [18]. As an alternative to chemical disinfectants, solar radiation is used due to the germicidal power of ultraviolet (UV) radiation and thermal heating [2]. While 2.5 h of exposure to simulated solar radiation resulted in a 5-log reduction in *Escherichia coli* or *Pseudomonas aeruginosa*, 8 h of treatment was ineffective against *A. polyphaga* cysts [19].

In addition, some studies proved that bacteria, when shielded by amoebae, resisted higher doses of conventional disinfectants compared to free bacteria. Kilvington and Price [20] and García et al. [21] found that *L. pneumophila* could resist several times higher NaOCl doses when protected by *A. polyphaga*. He et al. [22] proved that *Burkholderia* bacterium was not effectively inactivated by $UV_{254\ nm}$ while inside *Dictyostelium discoideum* cysts, observing only a 1-log reduction at the same UV dose (20 W.s/m^2) that could cause a 6-log reduction in the free bacteria.

There is a wide variety of environmental FLA that can reach artificial water systems, as well as a wide variety of endosymbiont bacteria that can undergo water treatments while protected inside amoebae. The potentially pathogenic bacteria carried by FLA are frequently more worrying than the amoeba itself, and may become more virulent during intra-amoebal life [6]. Additionally, FLA can transport antibiotic-resistant bacteria [23]. Thus, focusing disinfection research on these protected and hidden microorganisms becomes crucial. Moreover, the increase in reusing and regenerating water, particularly in regions with water scarcity, a situation exacerbated by climate change [1], reinforces the necessity to develop technologies capable of inactivating potential pathogens that may become recalcitrant in the urban water cycle.

Some alternative treatments have also been assessed, including ozone, chlorine dioxide, or monochloramine [16]. However, the combination of chlorine and solar radiation can become an interesting choice given that these are the most widely used water disinfection treatments worldwide [24] and their combination turns the disinfection process into an Advanced Oxidation Process (AOP). Oxidant species such as hydroxyl radicals ($^{\bullet}OH$), ozone (O_3), and chlorine radicals ($Cl^{\bullet}$, $Cl_2^{\bullet -}$, $ClO^{\bullet}$) can be generated in situ through chlorine photolysis at the UV wavelengths characteristic of natural sunlight [25]. These molecules are highly reactive and have a biocide effect [26]. Indeed, it has been demonstrated that sunlight-driven chlorine photolysis dramatically accelerates the inactivation of highly chlorine-resistant microorganisms such as *Cryptosporidium parvum* [24] and *Bacillus subtilis* endospores [27], attracting interest due to its efficiency and residual chlorine effect [26]. However, studies on the inactivation of amoebae by chlorine photolysis are limited, though interesting results have been found. Chauque and Rott [28] evaluated the effect of UV_{254nm} chlorine photolysis on *A. castellanii*, finding that cysts were inactivated after prolonged exposures (two rounds of 90 min) to 1.0 or 5.0 mg/L of NaClO photolysis.

Wang et al. [29] evaluated the effect of solar radiation/chlorine on *Dictyostelium discoideum* amoebae and *Burkholderia agricolaris* intracellular bacteria, finding a 5-log inactivation of both amoebae and bacteria after 20 min of 5 mg/L solar/chlorine treatment. Nevertheless, the effectiveness of chlorine photolysis against environmental endosymbiont bacteria has never been studied.

Acanthamoeba sp. is recognized as the amoeba genus most frequently found in the environment. In addition, it can cause human infections and can host numerous potentially pathogenic microorganisms, including intracellular bacteria [7], being one of the most resistant FLA genera reported [16]. Furthermore, results with *Acanthamoeba* can be extrapolated to other pathogenic protozoa that are equally or less resistant (and not as frequent as *Acanthamoeba*), such as *Cryptosporidium* spp., *Giardia duodenalis*, *Naegleria fowleri*, or *Entamoeba histolytica* oocysts [30]. Therefore, *Acanthamoeba* is a reliable model to simulate water treatment processes and evaluate the efficiency and usefulness of different disinfectants.

Given these considerations, the main objective of this study was to evaluate the effectiveness of the combination of NaClO (Cl_2) and solar radiation (SR) against *Acanthamoeba* (FLA) and their endosymbiont bacteria (EB). Treatments were developed against two environmental *Acanthamoeba* strains, one isolated from freshwater and one from chlorinated and solar-exposed water. Different chlorine concentrations, exposure times, and UV wavelengths were evaluated. This study represents the first disinfection investigation in which environmental endosymbiont bacteria carried by *Acanthamoeba* were evaluated.

2. Materials and Methods

2.1. Strain Isolation and FLA Inoculum

Two different environmental *Acanthamoeba* spp. strains were tested: P31 FLA (GenBank accession No. KY038362), isolated from the recreational water of an outdoor swimming pool in Zaragoza (Spain) [31], and C1-211 FLA, isolated from the freshwater of the Noguera Ribagorzana river in Lérida (Spain). The isolation procedure was performed as previously described [5]. The isolated C1-211 FLA strain was submitted to PCR and further sequentiation for genus and genotype identification, following the specific protocol for *Acanthamoeba* identification described by Schroeder et al. [32]. The sequence was further compared with the GenBank database thanks to the BLAST (Basic Local Alignment Search Tool of the National Center for Biotechnology Information, NCBI) bioinformatic tool. The C1-211 FLA sequence was registered in GenBank under the number OQ927217.

Both isolates grew in axenic conditions in a protease peptone, yeast extract, and glucose (BD Difco laboratories, MI, USA) medium (PPYG) at 30 °C. Before each treatment, FLA were harvested by centrifugation at 6000× *g* for 10 min from PPYG medium cultures and washed in a saline solution (0.9% NaCl, Panreac Química S.L.U., Barcelona, Spain).

2.2. Disinfection Treatments

Disinfection assays were carried out with 20 mL of the sample in sterile 50 mL quartz beakers with continuous stirring. The initial FLA concentration in the assays was adjusted to $1{\cdot}10^4$–$2{\cdot}10^4$ cells/mL. Five disinfection treatments were evaluated: chlorine (Cl_2), solar radiation with a wavelength range between 320 and 800 nm ($SR_{320–800nm}$), solar radiation with a wavelength range between 280 and 800 nm ($SR_{280–800nm}$), the combination of Cl_2 and $SR_{320–800nm}$ ($Cl_2/SR_{320–800nm}$), and the combination of Cl_2 and $SR_{280–800nm}$ ($Cl_2/SR_{280–800nm}$). Cl_2, $SR_{320–800nm}$, and $SR_{280–80nm}$ treatments were developed as gold standards [33].

Cl_2 disinfection assays were conducted in dark conditions and prepared by diluting a 10% *w*/*v* sodium hypochlorite solution (Panreac Química S.L.U., Barcelona, Spain). The assays for $SR_{280–800nm}$, $SR_{320–800nm}$, and their combination with Cl_2 ($Cl_2/SR_{320–800nm}$ and $Cl_2/SR_{280–800nm}$) were carried out in an Atlas Suntest CPS+/XLS+ solar chamber equipped with a xenon lamp. This system allowed the reproduction of natural sunlight conditions in the laboratory, covering wavelengths from 280 to 800 nm. A quartz filter and an additional glass filter Xenochrome 320 (Ameteck Instruments S.L., Barcelona, Spain) were used to

eliminate wavelengths below 320 nm in $SR_{320–800nm}$ and $Cl_2/SR_{320–800nm}$ assays. All the SR assays were conducted with a light intensity of 500 W/m^2, corresponding to 50% of the light intensity of the midday equatorial solar radiation [34]. The temperature was maintained below 30 °C during the experiments to ensure that only solar radiation (and not heating) played a role in the disinfection process.

According to the chlorine resistance of *Acanthamoeba* [16], the studied Cl_2 concentrations ranged between 1 and 500 mg/L of Cl_2. In accordance with WHO guidelines [1], the exposure time was set to 30 min. A control without Cl_2 in darkness was evaluated. Additionally, a control without Cl_2 under $SR_{320–800nm}$ and $SR_{280–800nm}$ was evaluated to assess the effect of SR. Aliquots were sampled at the beginning and during the disinfection assays to assess the survival of FLA and the endosymbiont bacteria (EB), pH (using a pH meter, GLP 21 Crison, Hach Lange Spain, Barcelona, Spain), and Cl_2 concentration. Assays were performed in duplicate.

Cl_2 exposure was quantified by the CT factor [35,36]. The residual concentration of Cl_2 was measured by two methodologies: for Cl_2 concentrations above 5 mg/L, sodium thiosulphate microtitration was applied to the sampled aliquots [37] using sodium thiosulphate (Panreac Química S.L.U., Barcelona, Spain), and for Cl_2 concentrations below 5 mg/L, the N,N-diethyl-p-phenylenediamine (DPD) methodology was applied [37] using the free chlorine reagent (Hannah Instruments, Eibar, Spain) and a JenwayTM 6305 UV/visible spectrophotometer (Fisher Scientific S.L., Madrid, Spain).

$SR_{280–800nm}$ and $SR_{320–800nm}$ exposures were quantified by the SR dose or fluence (F), which is directly related to the inactivation of microorganisms. As the light intensity applied was constant over time, the SR dose or fluence can be calculated as a product of the light intensity and the exposure time: F = light intensity (W/m^2) × time (s), in $W.s/m^2$ [35].

2.3. Determination of FLA and EB Inactivation

FLA survival was quantified using the most probable number (MPN) procedure. A sodium thiosulfate solution was added to the aliquots for chlorine neutralization. Ten microliters of the sampled aliquots and their dilutions (10^{-1} and 10^{-2}) was inoculated onto Non-Nutrient Agar (NNA, BD Difco laboratories, MI, USA) plates covered by heat-inactivated *Escherichia coli*. Each inoculation was performed in quintuplicate. Additionally, 100 µL of the sampled aliquots was inoculated in quintuplicate on a separate plate. The plates were incubated for 15 days at 30 °C and regularly observed under an optical microscope to determine the presence or absence of growth with a ZEISS-Axiostar plus microscope (Carl Zeiss Ibérica S.L., Madrid, Spain). Results were reported using an MPN table [38]. If no amoebic growth was detected in the 10 µL aliquots, then the 100 µL aliquots were checked for growth; no growth indicated total killing of the amoebae.

The inactivation of FLA was defined as a logarithmic reduction (N/N_0), where N_0 and N were the most probable number of viable cells before and after *t* time of the treatments, respectively. FLA inactivation was recorded as a function of chlorine concentration (mg/L). The necessary Cl_2 concentration for a 2-log ($[Cl_2]_{99\%}$) and a 3-log ($[Cl_2]_{99.9\%}$) inactivation was calculated from the linear regression section of the FLA inactivation curves. FLA inactivation by only Cl_2 was also recorded as a function of CT (mg.min/L) in Cl_2 assays. The necessary CT for a 2-log ($CT_{99\%}$) and a 3-log ($CT_{99.9\%}$) inactivation was calculated from the linear regression section. In the case of $SR_{280–800nm}$ and $Cl_2/SR_{280–800nm}$ assays, FLA inactivation was also recorded as a function of time (min) and fluence ($KW.s/m^2$). The necessary $SR_{280–800nm}$ fluence for a 2-log ($F_{99\%}$) and a 3-log ($F_{99.9\%}$) inactivation was calculated from the linear regression section of the FLA inactivation curves. FLA inactivation curves were obtained by Microsoft Excel 365 Software.

EB survival was determined semi-quantitatively. A sodium thiosulfate solution was added to the aliquots for chlorine neutralization. In total, 10 µL and 100 µL of the sampled aliquots throughout the experiments were inoculated onto Müeller Hinton agar plates (BD Difco laboratories, MI, USA). Each inoculation was performed in quintuplicate for the disinfection assays. The plates were incubated for 48 h at 37 °C and observed every 24 h

to determine the presence or absence of growth. EB survival was categorized into three groups: non-affected survival if bacteria growth was similar to control; affected survival if a reduction in bacteria growth was observed compared to control; and the inactivation of bacteria if no bacteria growth was detected after the treatment.

3. Results

3.1. Inactivation of Acanthamoeba by Conventional Treatments

To determine the disinfection effectiveness of Cl_2, FLA and EB inactivations were evaluated after 30 min of exposure to Cl_2 concentrations that varied from 1 to 500 mg/L (Figure 1a). Exposure to Cl_2 yielded a gradual *Acanthamoeba* C1-211 reduction to its total inactivation after exposures of 100 mg/L. In the case of *Acanthamoeba* P31, 250 mg/L of Cl_2 was necessary to achieve a 3-log reduction ($[Cl_2]_{99.9\%}$ in Table 1) and inactivate FLA. FLA inactivation by Cl_2 was also recorded as a function of CT (Figure 1b) to calculate the necessary CT values for a 2-log and a 3-log FLA inactivation (Table 1).

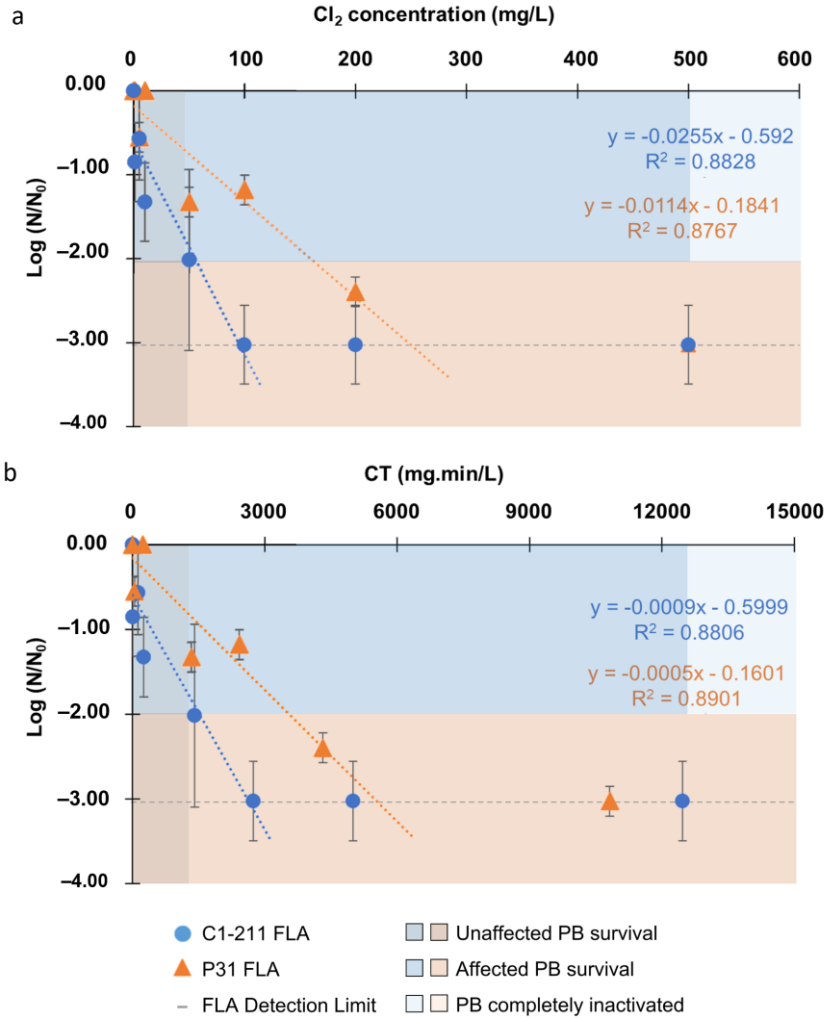

Figure 1. FLA and EB inactivation after 30 min of Cl_2 disinfection treatment at different (**a**) Cl_2 concentrations and (**b**) CT values. The darkest blue (C1-211) and orange (P31) background colors indicate that EB survival was not affected; intermediate shades of blue and orange colors indicate that EB survival was affected; and the lightest shades of blue and orange colors indicate that EB was totally inactivated.

Table 1. Required Cl_2 doses (mg/L), CT (mg.min/L), and F (KW.s/m^2) for achieving a 2-log (99%) and a 3-log (99.9%) inactivation of *Acanthamoeba* and EB.

	C1-211			P31		
Treatment	$[Cl_2]_{99\%}$	$[Cl_2]_{99.9\%}$	$[Cl_2]_{EB}$	$[Cl_2]_{99\%}$	$[Cl_2]_{99.9\%}$	$[Cl_2]_{EB}$
Cl_2	55	94	500	159	247	>500
$Cl_2/SR_{320–800nm}$	4	8	50	178	319	500
$Cl_2/SR_{280–800nm}$	-	-	5	1	4	100
Treatment	**$CT_{99\%}$**	**$CT_{99.9\%}$**	**CT_{EB}**	**$CT_{99\%}$**	**$CT_{99.9\%}$**	**CT_{EB}**
Cl_2	1556	2667	12,471	3680	5680	>10,820
Treatment	$F_{99\%}$	$F_{99.9\%}$	F_{EB}	$F_{99\%}$	$F_{99.9\%}$	F_{EB}
$SR_{320–800nm}$	>900	>900	>900	>900	>900	>900
$SR_{280–800nm}$	178	285	900	-	-	>900

To determine the disinfection effectiveness of SR, FLA and EB inactivations were assessed throughout the 30 min of SR exposure. Two wavelength ranges were compared: 280–800 nm (which included UV-A and UV-B radiation, Figure 2) and 320–800 nm (which included UV-A). Results showed that $SR_{320–800nm}$ alone did not result in FLA reduction after 30 min of exposure. In contrast, 10 min (fluence of 300 KW.s/m^2) of $SR_{280–800nm}$ alone completely inactivated C1-211 FLA. The necessary fluence for a 2-log and a 3-log C1-211 FLA inactivation was calculated from the linear section of the inactivation curve (Table 1). For P31 FLA, $SR_{280–800nm}$ alone caused a gradual 1.5-log reduction during the first 5 min, but FLA survival was sustained throughout the remainder of the experiment; hence, the necessary fluence for a 2-log or a 3-log reduction in P31 FLA remains unknown.

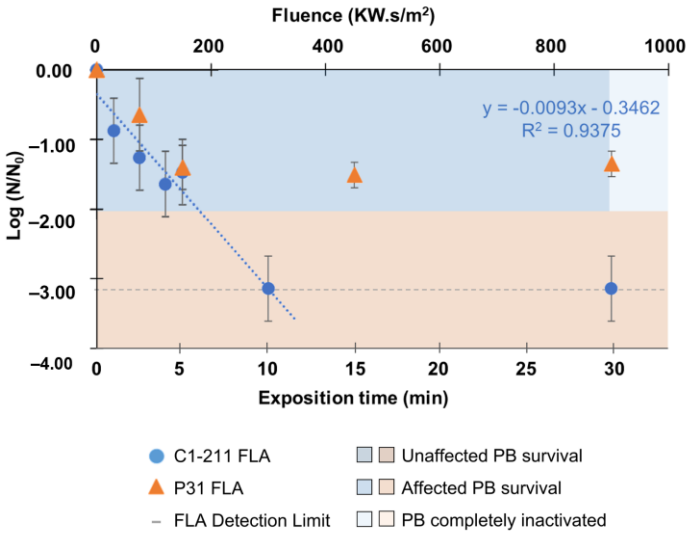

Figure 2. FLA and EB inactivation during 30 min of $SR_{280–800nm}$ disinfection treatment. The darkest blue (C1-211) and orange (P31) background colors indicate that EB survival was not affected; intermediate shades of blue and orange colors indicate that EB survival was affected; and the lightest shades of blue and orange colors indicate that EB was totally inactivated.

3.2. Inactivation of Acanthamoeba by Chlorine Photolysis

To assess the effectiveness of the combination of Cl_2 and SR against FLA and EB, the same wavelength ranges (280–800 nm and 320–800 nm) and Cl_2 concentrations (ranging from 1 to 500 mg/L) were compared after 30 min of treatment (Figure 3a,b). The combination of Cl_2 and simulated SR enhanced the reduction in C1-211 FLA using both wavelength ranges, while only the combination of Cl_2 and $SR_{280–800nm}$ enhanced P31 FLA inactivation. The combination of 5 mg/L of Cl_2 with both SR wavelength ranges resulted in a 3-log

reduction in C1-211 FLA (Figure 4a,b), reducing the necessary inactivating Cl_2 concentration by 20 times. In the case of P31, the FLA reduction obtained by the combination of Cl_2 and $SR_{320–800nm}$ was similar to that obtained by exposure to only Cl_2: approximately 300 mg/L for 30 min was necessary for a 3-log FLA reduction (Table 1). On the contrary, the combination of 5 mg/L of Cl_2 and $SR_{280–800nm}$ totally inactivated P31 FLA, reducing the required amount of Cl_2 by 50 times.

FLA inactivation was also assessed at different times during the disinfection assays combining Cl_2 and $SR_{280–800nm}$. When combining 1 or 5 mg/L of Cl_2 with simulated $SR_{280–800nm}$, C1-211 FLA reduction was not improved compared to $SR_{280–800nm}$ alone (Figure 4a). However, the combination of 50 mg/L of Cl_2 and simulated $SR_{280–800nm}$ accelerated the C1-211 FLA reduction compared to $SR_{280–800nm}$ alone, yielding a 3-log reduction after 5 min of exposure instead of 10 min. In the case of P31 FLA (Figure 4b), the combination of Cl_2 and simulated $SR_{280–800nm}$ improved P31 FLA reduction compared to Cl_2 or $SR_{280–800nm}$ alone: a 2-log, 3-log, and 4-log P31 FLA reduction was achieved combining $SR_{280–800nm}$ exposure with 1, 5, and 50 mg/L of Cl_2, respectively. Taking the results of both FLA strains together, the combination of 5 mg/L of Cl_2 and $SR_{280–800nm}$ for 10 min achieved a 3-log FLA inactivation for both *Acanthamoeba* strains studied, drastically reducing the necessary Cl_2 concentration for their inactivation by 20–50 times.

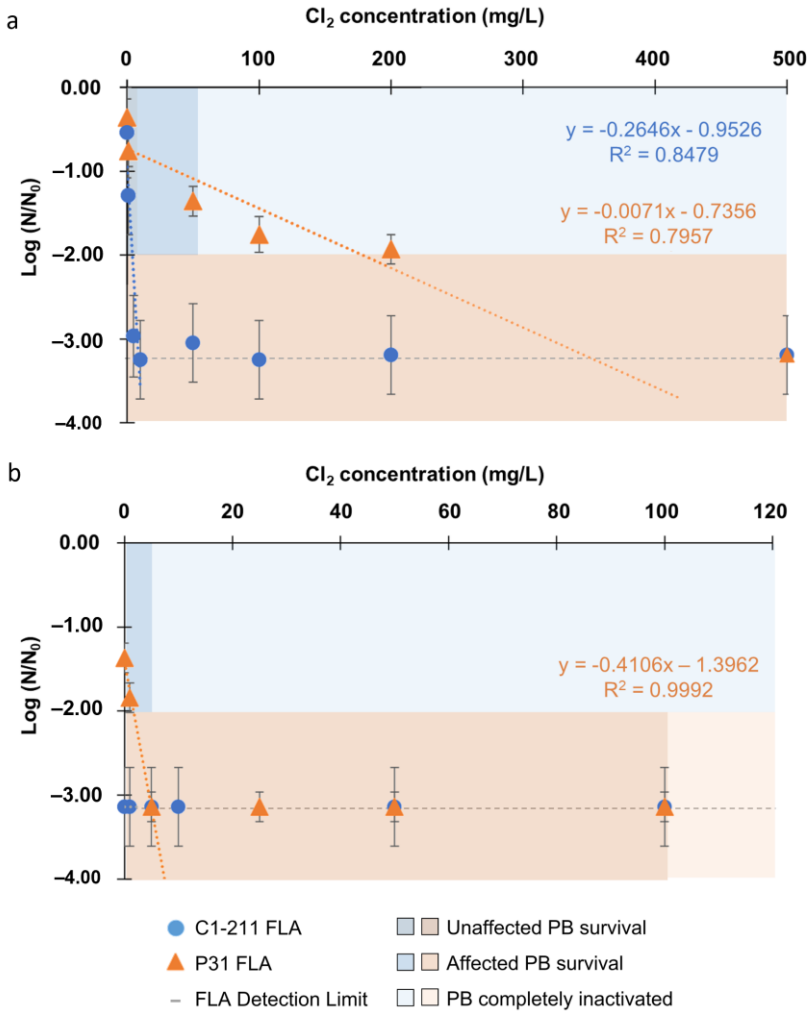

Figure 3. FLA and EB inactivation after 30 min of (**a**) the combination of Cl_2 and $SR_{320–800nm}$ and (**b**) the combination of Cl_2 and $SR_{280–800nm}$ at different Cl_2 concentrations. The darkest blue (C1-211) and orange (P31) background colors indicate that EB survival was not affected; intermediate shades of blue and orange colors indicate that EB survival was affected; and the lightest shades of blue and orange colors indicate that EB was totally inactivated.

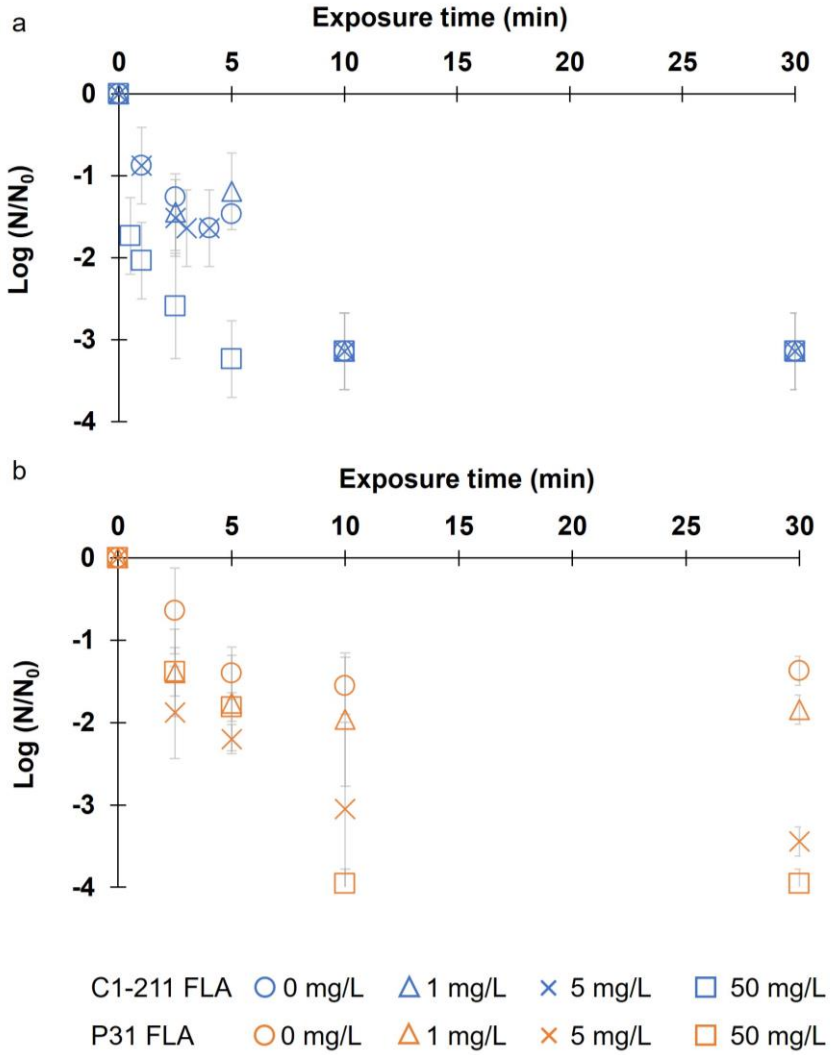

Figure 4. Inactivation of (**a**) C1-211 FLA and (**b**) P31 FLA along 30 min of combining Cl_2 and $SR_{280–800nm}$ with different Cl_2 concentrations.

3.3. Endosymbiont Bacteria Inactivation

To assess the disinfection efficiency of the treatments on the environmental endosymbiont bacteria protected by the studied FLA, EB survival was measured semi-quantitatively by determining the presence or absence of growth after the treatments. The survival of EB was categorized as non-affected survival, affected survival, or inactivation, as indicated by the colors in Figures 1–3. To compare the effectiveness of treatments, the necessary [Cl_2], CT, and F for BRA inactivation were described based on the conditions that resulted in a total absence of EB growth under the studied conditions (Table 1).

Cl_2 affected EB survival if the concentration was equal to or higher than 50 mg/L for both FLA (Figure 1a); EB survival remained unaffected at lower Cl_2 concentrations. To achieve complete bacterial growth inactivation, exposures of 500 mg/L for 30 min were necessary for C1-211, while higher Cl_2 doses might be necessary for the total inactivation of P31 EB. The CT value necessary for inactivating C1-211 EB is 12,471 mg.min/L (Table 1), four times higher than the CT value necessary for C1-211 FLA inactivation ($CT_{99.9\%}$). In the case of P31, the CT value necessary for P31 EB inactivation might be higher than 10,820 mg.min/L.

Similar to FLA, EB survival was unaffected after exposure to $SR_{320–800nm}$ alone, even at the highest SR exposures used (900 KW.s/m^2). However, $SR_{280–800nm}$ irradiation did affect EB. In the case of C1-211, $SR_{280–800nm}$ achieved the absence of EB growth after 30 min

of exposure (900 KW.s/m^2, three times higher fluence than C1-211 FLA). For P31, 30 min of exposure was not enough to achieve the total inactivation of bacterial growth (Figure 2).

The combination of Cl_2 and simulated SR dramatically reduced the required concentration of Cl_2 for inactivating C1-211 EB growth: from 500 mg/L of Cl_2 (Figure 1) to 50 mg/L if combining Cl_2 and simulated $SR_{320–800nm}$ (Figure 3a) and to 5 mg/L when combining it with $SR_{280–800nm}$ (Figure 3b). In the case of P31, doses of 500 mg/L might be necessary for inactivating EB growth when combining Cl_2 and simulated $SR_{320–800nm}$ (Figure 3a), whereas combining Cl_2 and simulated $SR_{280–800nm}$ (Figure 3b) required 100 mg/L of Cl_2 for inactivating P31 EB, reducing the necessary Cl_2 for P31 EB inactivation more than five times.

4. Discussion

4.1. Acanthamoeba Inactivation by Conventional Treatments: Chlorine and Solar Radiation

Chlorine is a widely used disinfectant, especially in drinking water processes, due to its efficiency against bacteria. However, higher doses than those typically used (1–5 mg/L) in freshwater or swimming pool water treatments are necessary to inactivate protozoans [2,35] such as *Cryptosporidium* spp., *Giardia* spp., or Free-Living Amoebae (FLA). Moreover, effective doses vary among genera; for instance, 1–7 mg/L of free chlorine during 5–30 min was cysticidal against *Naegleria* spp., while 10 mg/L of free and combined chlorine for 30 min was effective against *Vermamoeba vermiformis* [16]. *Acanthamoeba*, as revealed in the present study, was reported to be especially resistant to high chlorine doses [18].

The obtained results showed that the *Acanthamoeba* isolates studied were highly resistant to Cl_2, needing treatments of 100 mg/L and 250 mg/L for 30 min to achieve the total inactivation of C1-211 and P31, respectively (Figure 1a and Table 1). These doses are 100 times higher than those typically used for drinking water processes, explaining the presence of FLA in drinking water networks, recreational water [12], ornamental fountains [14], or even hospital water [13]. These findings align with Storey et al. [39], who reported that 100 mg/L of chlorine for 10 min was ineffective for inactivating *Acanthamoeba*. Nevertheless, the isolates studied here were more sensitive than others studied previously, such as *Acanthamoeba* spp. studied by Gabriel and Panaligan [18] that suffered less than a 2-log reduction after Cl_2 doses of 500 mg/L or some of the isolates evaluated by Coulon et al. [40] that survived after 2500 mg/L for 30 min. It should be noted that such resistances belong to cysts, which can be between 6 and 30 times more resistant to chlorine doses than trophozoites [41], while in this study, FLA inoculums contained both cysts and trophozoites.

The efficacy of chlorine as a disinfectant can be predicted by the CT value [42]. The results for both *Acanthamoeba* isolates studied (Table 1) are consistent with findings by Loret and Greub [15], who compiled FLA inactivation data from different studies and estimated that the $CT_{99\%}$ for *Acanthamoeba* cysts was between 1200 and 6500 mg.min/L of Cl_2. When compared with Dupuy et al. [41], both amoebae studied here exhibited greater resistance to chlorine, achieving a $CT_{99\%}$ of 865 mg.min/L for *Acanthamoeba* cysts.

Due to the generation of trihalomethanes and other potentially carcinogenic chlorine byproducts [43], the Cl_2 doses demonstrated as effective in this study are too high for practical use in real installations. Therefore, alternatives are necessary.

Solar radiation is used as a disinfectant due to the germicidal power of ultraviolet (UV) radiation (and thermal heating). This germicidal efficiency is highly dependent on the wavelength, with the UVC range (200–280 nm) being the most effective, followed by the UVB (280–320 nm) and UVA (320–400 nm). However, solar disinfection activity is limited to wavelengths between 290 and 400 nm, as the Earth's atmosphere naturally absorbs UVC and part of UVB radiation [2]. In this study, the disinfectant power of two solar radiation wavelength ranges was evaluated: $SR_{280–800nm}$ (part of UVB, UVA, and visible radiation) and $SR_{320–800nm}$ (UVA and visible radiation). These ranges represent direct exposure to SR and the exposure to SR through a glass or plastic reactor (as in SODIS processes), respectively. On the one hand, the UVB range directly acts on the

DNA of cells, inducing the formation of thymine dimers [44] that can inhibit the normal replication and transcription of DNA [17]. UVB can also damage microorganisms through photosensitization mechanisms that produce reactive oxygen species (ROS), affecting different cellular components, including DNA, indirectly. On the other hand, UVA can only indirectly damage DNA through ROS action; it can also increase the permeability of the cell [45] and affect vital compounds for cell metabolism and homeostasis [44].

Results revealed that $SR_{320–800nm}$ (fluences of 900 KW.s/m^2) was completely ineffective, while $SR_{280–800nm}$ totally inactivated C1-211 FLA after a fluence of 300 KW.s/m^2 and partially inactivated (1.5-log reduction) P31 FLA after 150 KW.s/m^2, maintaining this inactivation rate even after exposure to a fluence of 900 KW.s/m^2 (Figure 2). These results point out that the UVB range (280–320 nm) could cause a reduction in FLA, but only the UVA-induced oxygen reactive species were not effective enough to inactivate these FLA, though UVA could have a synergistic effect with UVB radiation effects during $SR_{280–800nm}$ assays. The 1.5-log P31 FLA reduction achieved and maintained after 5 min of $SR_{280–800nm}$ treatment might correspond with trophozoite inactivation. P31 cysts might remain viable after this exposure, agreeing with Lonnen et al. [19], who reported that solar disinfection (870 W/m^2 in the 300 nm–10 µm wavelength range) reduced the viability of *A. polyphaga* trophozoites but was ineffective against cysts.

It is difficult to compare these results with previous studies, as long as different UV radiation wavelengths and light intensities have been studied. Still, the results of $SR_{320–800nm}$ might align with those of Adan et al. [45], who reported less than a 1-log reduction in the trophozoites of an *Acanthamoeba* strain after 125 min of UVA irradiation. $F_{99\%}$ and $F_{99.9\%}$ values might help to compare with other studies, though differences in treatment conditions and whether cysts or trophozoites are studied should be noted (Table 1). In this context, C1-211 and P31 FLA appear to be more sensitive to $SR_{280–800nm}$ than the *Acanthamoeba* strains studied by Heaselgrave and Kilvington [46] and Lonnen et al. [19]. Heaselgrave and Kilvington [46] irradiated *A. castellanii* cysts with 550 W/m^2 of $SR_{290–800nm}$ and achieved a 2-log reduction after 2 h of treatment ($F_{99\%}$ = 3960 KW.s/m^2). Lonnen et al. [19] found that *A. polyphaga* trophozoites suffered a 2-log reduction after 2 h of 870 W/m^2 of $SR_{300nm–10\mu m}$ disinfection treatment ($F_{99\%}$ = 6264 KW.s/m^2), while cysts were barely reduced (less than 1-log reduction) after 8 h of solar disinfection (fluence of 28,800 KW.s/m^2).

Solar radiation, though considered an interesting alternative to chlorine for water disinfection as it avoids the formation of organochlorides [43], did not achieve the complete inactivation of the P31 *Acanthamoeba* strain studied, underlying the necessity of alternative disinfection processes.

4.2. Acanthamoeba Inactivation by Advanced Oxidation Process: Combination of Cl_2 and SR

Powerful oxidizing agents are generated during Advanced Oxidation Processes (AOPs). These highly reactive species have a biocide effect since they act non-selectively on biomolecules, compromising the survival of microorganisms [26]. Oxidant species such as ozone and hydroxyl radicals ($^{\bullet}OH$) can be generated in situ through Cl_2 photolysis at the UV wavelengths characteristic of natural sunlight [24]. Additionally, reactive chlorine species (RCS) such as $Cl^{\bullet}$, $Cl_2^{\bullet-}$, and $ClO^{\bullet}$ are formed during this process and may also contribute to microbial inactivation [29]. Indeed, sunlight-driven chlorine photolysis has already been demonstrated to dramatically accelerate the inactivation of highly chlorine-resistant microorganisms such as *Bacillus subtilis* endospores [27] and *Cryptosporidium parvum* [24]. Thus, the inactivation efficiency of the combination of Cl_2 and SR at two different wavelength ranges was evaluated.

Results of the combination of Cl_2 and SR assays (Figures 3 and 4) showed that *Acanthamoeba* reduction could be much more efficient than Cl_2 or SR treatments alone, especially when the $SR_{280–800nm}$ wavelength range was used. The combination of 5 mg/L of Cl_2 and $SR_{280–800nm}$ for 10 min achieved a 3-log reduction in both C1-211 and P31 FLA, reducing the necessary Cl_2 for their inactivation between 20 and 50 times. This presents an interest-

ing and attractive disinfection treatment for eliminating amoebae from water. Although $SR_{280–800nm}$ and 5 mg/L of $Cl_2/SR_{280–800nm}$ treatment were equally efficient against C1-211 FLA, maintaining a residual disinfection effect after the treatment, as in the case of $Cl_2/SR_{280–800nm}$, may produce beneficial results [26]. These results agree with Wang et al. (2023), who obtained similar efficiencies when testing the combination of 5 mg/L of Cl_2 and solar radiation against *Dictyostelium discoideum* amoebae: a 3-log reduction after 10 min of exposure that increased to a 5-log reduction when the exposure time increased to 20 min.

In comparison, the combination of Cl_2 and $SR_{320–800nm}$ was effective in removing C1-211 FLA, but it was counterproductive against P31 FLA. Taking into account that chlorine photolysis combines multiple mechanisms to inactivate pathogens, (1) a direct reaction with $HOCl/OCl^-$, (2) direct photolysis by UV irradiation, and (3) oxidizing molecule attacks (ROS, RCS, and O_3) [25], variations between combining Cl_2 with $SR_{280–800nm}$ and $SR_{320–800nm}$ might be related to different disinfection mechanisms implied. The direct photolysis of chlorine and the subsequent formation of reactive oxidant species are dependent on pH and wavelength. Considering the pH of the assay solutions (Table 2) and the HOCl acid dissociation constant (pKa = 7.5), OCl^- might be the predominant chlorine species present in most of the assays combining Cl_2 and SR. The two shifting chlorine species ($HOCl/OCl^-$) have different UV–visible absorption spectra: OCl^- has its maximum absorption coefficient at 292 nm. Hence, the irradiation of OCl^- at 280–800 nm (during the assays that combine Cl_2 and $SR_{280–800nm}$) might mainly produce $^{\bullet}OH$ thanks to successive reactions (1–6). At wavelengths higher than 320 nm, as those irradiated during the assays that combine Cl_2 and $SR_{320–800nm}$ and after successive reactions (7–8), ozone is principally produced [25,27]. Looking at the results, $^{\bullet}OH$ radicals seem to be a more efficient inactivation route than ozone for C1-211 and P31 FLA inactivation.

$$HOCl + hv \rightarrow {}^{\bullet}OH + Cl^{\bullet} \quad (1)$$

$$OCl^- + hv \rightarrow O^{-\bullet} + Cl^{\bullet} \quad (2)$$

$$OCl^- + hv \rightarrow Cl^- + O(1D) \quad (3)$$

$$O(1D) + H_2O \rightarrow 2\ {}^{\bullet}OH \quad (4)$$

$$Cl^{\bullet} + H_2O \rightarrow HOCl^{-\bullet} + H^+ \quad (5)$$

$$HOCl^{-\bullet} \rightarrow {}^{\bullet}OH + Cl^- \quad (6)$$

$$OCl^- + hv \rightarrow O(3P) + Cl^- \quad (7)$$

$$O(3P) + O_2 \rightarrow O_3 \quad (8)$$

$$Cl^{\bullet} + OCl^- \rightarrow ClO^{\bullet} + Cl^- \quad (9)$$

$$Cl^{\bullet} + Cl^- \rightarrow {Cl_2}^{-\bullet} \quad (10)$$

Table 2. pH of Cl_2, $Cl_2/SR_{280–800nm}$, and $Cl_2/SR_{320–800nm}$ assays at different times.

Initial Concentration (mg Cl_2/L)	Cl_2		$Cl_2/SR_{280–800nm}$			$Cl_2/SR_{320–800nm}$	
	0 min	30 min	0 min	5 min	10 min	0 min	30 min
0	7.0	7.0	7.0	7.0	7.1	7.0	7.4
1	7.0	7.1	nd	nd	nd	7.1	7.4
5	8.4	8.2	8.4	8.6	9.5	8.6	7.4
10	8.6	7.6	nd	nd	nd	8.7	8.2
50	9.9	9.0	9.9	10.0	10.3	9.9	9.0
100	10.3	9.6	nd	nd	nd	10.3	9.8

Note: nd—not determined.

RCS formed during $Cl_2/SR_{280–800nm}$ and $Cl_2/SR_{320–800nm}$ treatments (reactions 1, 2, 9, 10) might also contribute to microbial inactivation, though it is not clear to what extent

as long as $Cl^{\bullet}$ reacts more selectively than $^{\bullet}OH$. In addition, if $Cl^{\bullet}$ production varies according to the irradiated wavelength, this has not been comprehensively studied yet [25]. Hence, the potential microbial inactivation role of RCS in the studied treatments remains unknown. Since this was not the main objective of the present work, further fundamental experimentation would be necessary to better understand RCS disinfection power during chlorine photolysis.

Forsyth et al. [27] suggested activating chlorine with UVA light as an attractive strategy to enhance the inactivation of chlorine-resistant pathogens. However, lower wavelengths (as those used during the combination of Cl_2 and $SR_{280–800nm}$) seem necessary for the effective inactivation of *Acanthamoeba*, even in the presence of Cl_2. In this sense, Chauque and Rott [28] evaluated the disinfection capacity of Cl_2/UV_{254nm} against an *A. castellanii* strain. They found that low Cl_2 concentrations (1–8 mg/L) during long UV_{254nm} (2.43 W/m^2) exposure times (two rounds of 90 min) were effective in inactivating both trophozoites and cysts. Although low Cl_2 doses were used to reduce the cost of water treatment, high energy input was necessary to cover those exposure times. Depending on water use and point-of-use requirements, such long times could not be feasible. Also, whether bacteria protected inside the amoeba could be able to survive after that treatment was not studied and remains unknown.

4.3. Inactivation of the Endosymbiont Bacteria Protected Inside Acanthamoeba

Free-Living Amoebae were reported to be protectors of a wide variety of microorganisms (viruses, bacteria, and fungi), making it possible to protect potentially pathogenic bacteria such as *Mycobacterium* spp., *Vibrio cholerae*, *Pseudomonas* spp., *Helicobacter pylori*, *Listeria monocytogenes*, or the most known and studied, *Legionella pneumophila* [6,7]. Amoebae undergo a dormant stage called the cyst when environmental conditions are not comfortable for their active form. This encystation process involves the production of a robust, thick wall that confers huge resistance to the amoebae against disinfectants. The cystic wall of *Acanthamoeba* is a double-layered wall composed of the ectocyst, which is mostly composed of proteins and polysaccharides, and the endocyst, mostly composed of cellulose. Higher cellulose content is associated with increased resistance to biocides [8]. While much is still to be learned about amoebic cysts, it is known that their composition varies among strains, leading to variations in resistance against disinfection treatments [40].

Although several studies have reported that the cystic stage is very resistant to harsh conditions and disinfectants [16], few have explored their capacity to protect the microorganisms inside during these treatments. Some studies have shown that bacteria are more resistant to disinfection treatments in the presence of amoebae than in their absence. Nevertheless, these findings were often attributed to phenotypical bacterial changes [22,47] or the scattering of UV light by amoebae [45], rather than to amoeba protection. Few studies evaluated the inactivation capacity of disinfectants against bacteria protected by amoeba, and in any case, amoebae were in vitro-infected, and conventional disinfectants such as chlorine [20], monochloramine, chlorine dioxide [47], or UV_{254nm} [22] were evaluated. Only Wang et al. [29] evaluated the inactivation capacity of chlorine photolysis against bacteria in vitro-internalized inside amoebae. Nevertheless, this study represents the first instance of evaluating the environmental endosymbiont bacteria protected and carried by amoebae among disinfection studies.

As a general trend, results showed that exposure doses that compromised FLA survival were insufficient for EB inactivation. EB survived even when FLA were inactivated (Table 1), proving that amoebae confer great protection to microorganisms within them, agreeing with reports by He et al. [22].

In the case of Cl_2 treatments (Figure 1a), EB might remain unaffected while both trophozoites and cysts remain barely unaffected (at Cl_2 concentrations lower than 50 mg/L). Bacteria inside trophozoites might start to be affected when Cl_2 concentrations are high enough to penetrate trophozoites, but cyst structures might remain intact even when inactivated [22], protecting bacteria inside at Cl_2 concentrations several times higher (Table 1).

Cl_2 doses above 500 mg/L (CT above 12,000 mg.min/L) were necessary to totally inactivate C1-211 EB, while bacteria protected by P31 FLA require higher Cl_2 doses. These results disagree with Wang et al. [29], who reported similar inactivation levels of *Dictyostelium discoideum* amoeba and *Burkholderia agricolaris* bacteria protected inside; however, this might be due to the high chlorine sensitivity of the amoeba ($CT_{99.9\%}$ of 40 mg.min/L) or to a different relationship between *D. discoideum* and its associated bacteria [48] compared to *Acanthamoeba* [6]. Otherwise, these results agree with García et al. [21], who reported that *L. pneumophila* could resist 1024 mg/L of NaOCl when protected by *A. polyphaga* ATCC 50998. It should be considered that the basic pH due to such high chlorine concentrations (Table 2) could also affect FLA survival, though according to Khan [49], *Acanthamoeba* can grow at pH ranging from 4 to 12.

Like FLA, $SR_{320–800nm}$ alone was unable to affect EB survival. This agrees with reports by Adan et al. [45], who found that *Escherichia coli* was barely unaffected after 150 min of UVA exposure if *Acanthamoeba* was also present. Conversely, $SR_{280–800nm}$ inactivated C1-211 EB growth after 30 min of exposure (900 KW.s/m^2), a three times higher dose than the one necessary for C1-211 FLA inactivation (Figure 2). He et al. [22] also reported that FLA were able to protect EB inside even after being inactivated by UV_{254nm}. He et al. [22] observed that cysts were not broken or damaged after UV_{254nm} radiation, suggesting that cysts can absorb and screen UV light, letting only a fraction of UV light reach the internalized bacteria. In the case of P31, $SR_{280–800nm}$ affected the bacteria protected, but some EB still survived after 900 KW.s/m^2, not achieving total bacteria inactivation. This might be related to the fact that such fluence was not enough to inactivate the amoebae that protected them either.

The combination of Cl_2 and SR, as conventional disinfection treatments, required higher doses for EB than for FLA inactivation, reinforcing the bacteria-protecting role of amoebae. The combination of Cl_2 and $SR_{320–800nm}$ required 10 times higher Cl_2 doses to inactivate C1-211 EB compared to FLA (50 vs. 5 mg/L, respectively). This disinfection treatment was not effective against P31 EB as long as it was not effective against P31 FLA either. In the case of combining Cl_2 and $SR_{280–800nm}$, 5 mg/L of Cl_2 was also efficient to inactivate C1-211 EB, but it was necessary to increase the exposure time from 10 min (necessary for inactivating C1-211 FLA) to 30 min, while increasing the Cl_2 dose 20 times was required to inactivate P31 EB (from 5 to 100 mg/L, compared with P31 FLA). This might be due to the cyst wall, which might act as a highly resistant shell, providing a protective environment inside, whether the cyst is active or inactive. The cyst wall resistance is directly related to its composition and the thickness of the layers, issues that might vary depending on the FLA strains but also on the culture and environmental conditions [8], potentially explaining differences in resistance to disinfection treatments between C1-211 and P31.

Comparing with treatments alone, the combination of Cl_2 and SR and the oxidizing species (ROS, RCS, and O_3) generated resulted in more efficient disinfection treatments, particularly $Cl_2/SR_{280–800nm}$, reducing several times the disinfectant doses necessary for EB inactivation. The combination of Cl_2 and $SR_{320–800nm}$ required 10 times lower Cl_2 doses to inactivate C1-211 EB compared to Cl_2 treatment (from 500 to 50 mg/L). This treatment, equivalent to adding chlorine to a solar disinfection (SODIS) process, though effective against C1-211 EB, did not achieve EB inactivation for P31 under the conditions studied, which is in accordance with FLA results. In the case of combining Cl_2 and $SR_{280–800nm}$, as applied to FLA, it proved to be a more efficient process than Cl_2 or SR alone against EB. $Cl_2/SR_{280–800nm}$ achieved the total inactivation of EB, reducing between 5 and 10 times the necessary Cl_2 dose. The enhancement in disinfectant efficiency through chlorine photolysis was also reported by Chauque and Rott [28] inactivating *Acanthamoeba castellanii*, Wang et al. [29] inactivating *D. discoideum* and *B. agricolaris*, and Zhou et al. [24] inactivating *Cryptosporidium parvum* oocysts, among others. Thus, results pose chlorine photolysis as an attractive disinfection process for further research. Cl_2 doses and exposure time optimizations could drive ready-to-use disinfection treatments tailored to specific water uses.

The generation of byproducts, such as trihalomethanes or haloacetic acids, from dissolved organic matter (DOM) during chlorination and chlorine photolysis has been frequently studied. While it is true that chlorine photolysis can reduce byproduct generation compared to chlorination processes by reducing DOM concentration, chlorine doses, and exposure time [25], further studies will be necessary to implement this treatment and prevent undesirable organic byproducts' formation.

Acanthamoeba can host a wide variety of bacteria within them [6]. Some of the endosymbiont bacteria carried by *Acanthamoeba* either do not grow under the conditions used in this study or are even non-culturable, requiring the use of reference bacteria to facilitate the visualization of the disinfection results. In this case, bacteria capable of growing rapidly in a nutrient medium and under an aerobic atmosphere were selected as references. Nevertheless, this study underlines the significant role amoebae can play in protecting and transporting potential pathogens throughout the water network.

4.4. Effect of Water Sources: Acanthamoeba Isolated from Natural vs. Artificial Water Sources

Differences in disinfection resistance were observed among the studied amoebae. Several studies have previously documented variations in resistance to disinfectants between different amoeba genera and strains. These differences are usually related to different cystic wall compositions [41]. Water sources from which amoebae are isolated might also influence disinfectant resistance, given that amoebae are adapted to different environmental conditions. As P31 has already overcome several disinfection treatments and inhabits an artificial water system such as an outdoor swimming pool, where it is constantly exposed to solar radiation and chlorine presence, it can be expected to be more resistant to all the treatments evaluated. Indeed, it agrees with Coulon et al. [40], who also reported higher resistance to disinfectants in the case of *Acanthamoeba* strains isolated from hospital water compared to *Acanthamoeba* strains isolated from river water.

The higher resistance to Cl_2 could be attributed to the constant presence of chlorine in the swimming pool where P31 FLA was isolated. Regarding solar radiation, since both amoebae were isolated from outdoor water environments reached by solar radiation, the differences found are likely to be due to the lower turbidity of the outdoor swimming pool where P31 FLA inhabited compared to the river where C1-211 FLA inhabited. In freshwater, numerous particles and microorganism aggregates can cause turbidity and scatter UV light [35] and thus, C1-211 FLA might be less accustomed to direct solar radiation. Given that P31 FLA was more resistant to Cl_2 and SR treatments, it was also expected to be more resistant to their combination. What raises significant concern is that the more resistant the *Acanthamoeba* strain was, the higher the disinfectant doses were required to inactivate their EB.

Based on the differences found, water disinfection protocols should be tailored to the source and intended use of the water. Freshwater or reclaimed water might contain amoebae similar to C1-211, and depending on its further use, a treatment capable of inactivating environmental FLA from freshwater and their protected bacteria should be used as a preventive measure (according to results, disinfecting with 5 mg/L of $Cl_2/SR_{280–800nm}$ for 30 min). In contrast, for water uses where the need for complete disinfection becomes imperative, such as hospitals or refrigeration towers, a more aggressive treatment would become necessary for inactivating FLA that are adapted to artificial water systems and their intracellular bacteria, such as *Acanthamoeba* P31 (according to results, 100 mg/L of $Cl_2/SR_{280–800nm}$ for 30 min). As these conditions are too demanding, further studies with longer exposure times and lower chlorine concentrations, as Chauque and Rott [28] recommended, are necessary.

This study, by comparing amoebae strains isolated from different water sources, provides insight into the importance of preventive measures for ensuring water quality. Environmental amoebae from freshwater, such as C1-211 FLA, can be very resistant to conventional treatments and easily reach artificial water systems. There, the endosymbiont bacteria carried by amoebae can be released and colonize the water system, posing a

health threat to exposed individuals. If additional FLA-ineffective treatments are included downstream, such as chlorination at swimming pools, the most resistant strains will be selected and will colonize the water system (as P31 FLA), making future efficient disinfection extremely difficult and leading to possible recurrent outbreaks of, e.g., *Legionella pneumophila* [13] or antibiotic-resistant bacteria [23]. If FLA-efficient disinfection treatments were established at water treatment plants, FLA populations in human-made water systems could be reduced. As it can be too expensive and difficult to include such treatments for large amounts of water, more aggressive treatments for lower flows would be convenient at specific points of water use where more sensitive people can be exposed, such as hospitals or swimming pools for children and elderly people.

In this context, $Cl_2/SR_{280–800nm}$ emerges as an interesting point-of-use treatment widely applicable since both chlorine and solar radiation are the most extended disinfection treatments worldwide, especially in developing areas [27], and would improve protection against other pathogens too [30]. Nevertheless, dissolved organic matter (DOM) should be considered as it may promote the formation of harmful byproducts when reacting with chlorine, determining the viability of the process. In this regard, the treatment may be more suitable for low-DOM waters [24,27], and further studies should be developed considering this issue.

As chlorine is the baseline disinfectant due to its high efficiency in pathogen removal cost-effectiveness, the results and methodology presented herein can serve as a valuable reference for further research aimed at optimizing the present conditions for a ready-to-use point-of-use treatment.

5. Conclusions

- Conventional chlorine and solar radiation disinfection treatments required high disinfectant doses and were not effective against the endosymbiont bacteria protected by the amoebae studied.
- The combination of Cl_2 and $SR_{320–800nm}$ was effective against the freshwater *Acanthamoeba* strain and its endosymbiont bacteria, reducing 10 times the required Cl_2 dose. Additionally, the Cl_2 and $SR_{280–800nm}$ combination was effective against both *Acanthamoeba* and their endosymbiont bacteria, reducing between 5 and 100 times the necessary Cl_2 dose.
- The inactivation of EB requires the prior inactivation of *Acanthamoeba* cysts, and because of that, the disinfectant doses for efficient disinfection were up to 20 times greater than those used for cyst inactivation.
- The *Acanthamoeba* strain isolated from pool water (P31) and, consequently, the EB protected inside showed considerable higher resistance to the combination of Cl_2 and $SR_{280–800nm}$ compared to the *Acanthamoeba* strain isolated from freshwater (C1-211), suggesting that higher disinfectant doses may be required to eliminate amoebae previously exposed to chlorine and solar radiation.

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References

1. World Health Organization. *Guidelines for Drinking-Water Quality, Fourth ed.: Incorporating the First and Second Addenda*; World Health Organization: Geneva, Switzerland, 2022; ISBN 978-92-4-004506-4. Available online: https://www.who.int/publications/i/item/9789240045064 (accessed on 28 December 2023).
2. Pichel, N.; Vivar, M.; Fuentes, M. The problem of drinking water access: A review of disinfection technologies with an emphasis of disinfection technologies with an emphasis on solar treatment methods. *Chemosphere* **2019**, *218*, 1014–1030. [CrossRef]
3. Benito, M.; Menacho, C.; Chueca, P.; Ormad, M.P.; Goñi, P. Seeking the reuse of effluents and sludge from conventional wastewater treatment plants: Analysis of the presence of intestinal protozoa and nematode eggs. *J. Environ. Manag.* **2020**, *261*, 110268. [CrossRef] [PubMed]
4. Visvesvara, G.S.; Moura, H.; Schuster, F.L. Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. *FEMS Immunol. Med. Microbiol.* **2007**, *50*, 1–26. [CrossRef]
5. Garcia, A.; Goñi, P.; Cieloszyk, J.; Fernandez, M.T.; Calvo-Beguería, L.; Rubio, E.; Fillat, M.F.; Peleato, M.L.; Clavel, A. Identification of Free-Living Amoebae and Amoeba-Associated Bacteria from Reservoirs and Water Treatment Plants by Molecular Techniques. *Environ. Sci. Technol.* **2013**, *47*, 3132–3140. [CrossRef] [PubMed]
6. Greub, G.; Raoult, D. Microorganisms Resistant to Free-Living Amoebae. *Clin. Microbiol. Rev.* **2004**, *17*, 413–433. [CrossRef] [PubMed]
7. Goñi, P.; Fernández, M.T.; Rubio, E. Identifying endosymbiont bacteria associated with free-living amoebae. *Environ. Microbiol.* **2014**, *16*, 339–349. [CrossRef] [PubMed]
8. Garajová, M.; Mrva, M.; Vaškovicová, N.; Martinka, M.; Melicherová, J.; Valigurová, A. Cellulose fibrils formation and organisation of cytoskeleton during encystment are essential for *Acanthamoeba* cyst wall architecture. *Sci. Rep.* **2019**, *9*, 4466. [CrossRef]
9. Marín, I.; Goñi, P.; Lasheras, A.M.; Ormad, M.P. Efficiency of a Spanish wastewater treatment plant for removal potentially pathogens: Characterization of bacteria and protozoa along water and sludge treatment lines. *Ecol. Eng.* **2015**, *74*, 28–32. [CrossRef]
10. Mosteo, R.; Ormad, M.P.; Goñi, P.; Rodríguez-Chueca, J.; García, A.; Clavel, A. Identification of pathogen bacteria and protozoa in treated urban wastewaters discharged in the Ebro River (Spain): Water reuse possibilities. *Water Sci. Technol.* **2013**, *68*, 575–583. [CrossRef]
11. Gomes, T.S.; Vaccaro, L.; Magnet, A.; Izquierdo, F.; Ollero, D.; Martínez-Fernández, C.; Mayo, L.; Moran, M.; Pozuelo, M.J.; Fenoy, S.; et al. Presence and interaction of free-living amoebae and amoeba-resisting bacteria in water from Drinking Water Treatment Plants. *Sci. Total Environ.* **2020**, *719*, 137080. [CrossRef]
12. Fernández, M.T. Molecular Characterization of Free-Living Amoebae and Identification of Other Parasites from the Water Network of Zaragoza District: Association with Other Microorganisms and Risks for Public Health. (Caracterización Molecular de Amebas de Vida Libre e Identificación de Otros Parásitos en Aguas de Red de la Provincia de Zaragoza: Asociación con Otros Microorganismos y Riesgos para la Salud Pública). Ph.D. Thesis, University of Zaragoza, Zaragoza, Spain, 2014.
13. Nisar, M.A.; Ross, K.E.; Brown, M.H.; Bentham, R.; Hinds, J.; Whiley, H. Molecular screening and characterization of *Legionella pneumophila* associated free-living amoebae in domestic and hospital water systems. *Water Res.* **2022**, *226*, 119238. [CrossRef]
14. Di Filippo, M.M.; Santoro, M.; Lovreglio, P.; Monno, R.; Capolongo, C.; Calia, C.; Fumarola, L.; D'Alfonso, R.; Berrilli, F.; Di Cave, D. Isolation and molecular characterization of freeliving amoebae from different water sources in Italy. *Int. J. Environ. Res. Public Health* **2015**, *12*, 3417–3427. [CrossRef]
15. Loret, J.F.; Greub, G. Free-living amoebae: Biological by-passes in water treatment. *Int. J. Hyg. Environ. Health* **2010**, *213*, 167–175. [CrossRef] [PubMed]
16. Thomas, V. Sensitivity and Resistance of Protozoa to Microbicides. In *Principles and Practice of Disinfection, Preservation and Sterilization*; Fraise, A.P., Maillard, J.Y., Sattar, S.A., Eds.; Blackwell Publishing Ltd.: Hoboken, NJ, USA, 2013; pp. 155–177.
17. Dalrymple, K.; Stefanakos, W.; Trotz, M.A.; Goswami, D.Y. A review of the mechanisms and modeling of photocatalytic disinfection. *Appl. Catal. B Environ.* **2010**, *98*, 27–38. [CrossRef]
18. Gabriel, A.A.; Panaligan, D.C. Heat and chlorine resistance of a soil *Acanthamoeba* sp. cysts in water. *J. Appl. Microbiol.* **2020**, *129*, 453–464. [CrossRef]
19. Lonnen, J.; Kilvington, S.; Kehoe, S.C.; Al-Touatic, F.; McGuiganc, K.G. Solar and photocatalytic disinfection of protozoan, fungal and bacterial microbes in drinking water. *Water Res.* **2005**, *39*, 877–883. [CrossRef] [PubMed]
20. Kilvington, S.; Price, J. Survival of *Legionella pneumophila* within cysts of *Acanthamoeba polyphaga* following chlorine exposure. *J. Appl. Bacteriol.* **1990**, *68*, 519–525. [CrossRef] [PubMed]

21. Garcia, M.T.; Jones, S.; Pelaz, C.; Millar, R.D.; Kwaik, Y.A. *Acanthamoeba polyphaga* resuscitates viable non-culturable *Legionella pneumophila* after disinfection. *Environ. Microbiol.* **2007**, *9*, 1267–1277. [CrossRef]
22. He, Z.; Wang, L.; Ge, Y.; Zhang, S.; Tian, Y.; Yang, X.; Shu, L. Both viable and inactivated amoeba spores protect their intracellular bacteria from drinking water disinfection. *J. Hazard. Mater.* **2021**, *47*, 126006. [CrossRef] [PubMed]
23. Muchesa, P.; Bartie, C.; Barnard, T.G. Free-Living Amoebae and Associated Drug-Resistant Bacteria in Surface Waters: A Public Health Concern? In *Parasite and Disease Spread by Major Rivers on Earth. Parasitology Research Monographs*; Mehlhorn, H., Klimpel, S., Eds.; Springer: Cham, Switzerland, 2019; Volume 12, pp. 221–236. [CrossRef]
24. Zhou, P.; Di Giovanni, G.D.D.; Meschke, J.S.; Dodd, M.C. Enhanced Inactivation of *Cryptosporidium parvum* Oocysts during Solar Photolysis of Free Available Chlorine. *Environ. Sci. Technol. Lett.* **2014**, *1*, 453–458. [CrossRef]
25. Remucal, C.K.; Manley, D. Emerging investigators series: The efficacy of chlorine photolysis as an advanced oxidation process for drinking water treatment. *Environ. Sci. Water Res. Technol.* **2016**, *2*, 565–579. [CrossRef]
26. Khajouei, G.; Finkle, H.O.; Lin, L.S. UV/chlorine advanced oxidation processes for degradation of contaminants in water and wastewater: A comprehensive review. *J. Environ. Chem. Eng.* **2022**, *10*, 107508. [CrossRef]
27. Forsyth, J.E.; Zhou, P.; Mao, Q.; Asato, S.S.; Meschke, J.S.; Dodd, M.C. Enhanced inactivation of *Bacillus subtilis* spores during solar photolysis of free available chlorine. *Environ. Sci. Technol.* **2013**, *47*, 12976–12984. [CrossRef]
28. Chauque, B.J.M.; Rott, M.B. Photolysis of sodium chloride and sodium hypochlorite by ultraviolet light inactivates the trophozoites and cysts of *Acanthamoeba castellanii* in the water matrix. *J. Water Health* **2021**, *19*, 190–202. [CrossRef]
29. Wang, L.; Mai, Y.; Li, S.; Shu, L.; Fang, J. Efficient inactivation of amoeba spores and their intraspore bacteria by solar/chlorine: Kinetics and mechanisms. *Water Res.* **2023**, *242*, 120288. [CrossRef]
30. Chauque, B.J.M.; Benetti, A.D.; Corçao, G.; Silva, C.E.; Gonçalves, R.F.; Rott, M.B. A new continuous-flow solar water disinfection system inactivating cysts of *Acanthamoeba castellanii*, and bacteria. *Photochem. Photobiol. Sci.* **2021**, *20*, 123–137. [CrossRef]
31. Ortillés, A.; Goñi, P.; Rubio, E.; Sierra, M.; Gámez, E.; Fernandez, M.T.; Benito, M.; Cristóbal, J.A.; Calvo, B. A rabbit model of *Acanthamoeba* keratitis: Use of infected soft contact lenses after corneal epithelium debridement with a diamond burr. *Investig. Ophthalmol. Vis. Sci.* **2017**, *58*, 1218–1227. [CrossRef] [PubMed]
32. Schroeder, J.M.; Booton, G.C.; Hay, J.; Niszl, I.A.; Seal, D.V.; Markus, M.B.; Fuerst, P.A.; Byers, T.J. Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoebae* from humans with keratitis and from sewage sludge. *J. Clin. Microbiol.* **2001**, *39*, 1903–1911. [CrossRef] [PubMed]
33. Claassen, J.A.H.R. The gold standard: Not a golden standard. *BMJ* **2005**, *330*, 1121. [CrossRef]
34. Yuranova, T.; Enea, O.; Mielczarski, E.; Mielczarski, J.; Albers, P.; Kiwi, J. Fenton immobilized photo-assisted catalysis through a Fe/C structured fabric. *Appl. Catal. B. Environ.* **2004**, *49*, 39–50. [CrossRef]
35. United States Environmental Protection Agency. *Alternative Disinfectants and Oxidants Guidance Manual*; Office of Water: Washington, DC, USA, 1999; EPA 815-R-99-014. Available online: https://www.epa.gov/ (accessed on 20 December 2023).
36. Kahler, A.M.; Cromeans, T.L.; Roberts, J.M.; Hill, V.R. Effects of source water quality on chlorine inactivation of adenovirus, coxsackievirus, echovirus and murine norovirus. *Appl. Environ. Microbiol.* **2010**, *76*, 5159–5164. [CrossRef]
37. American Public Health Association. *Standard Methods for the Examination of Water and Wastewater*, 21st ed.; American Public Health Association: Washington, DC, USA, 2005; ISBN 08-7553-047-8.
38. Beattie, T.K.; Seal, D.V.; Tomlinson, A.; McFadyen, A.K.; Grimason, A.M. Determination of amoebicidal activities of multipurpose contact lens solutions by using a most probable number enumeration technique. *J. Clin. Microbiol.* **2003**, *41*, 2992–3000. [CrossRef]
39. Storey, M.V.; Winiecka-Krusnelli, J.; Ashbolt, N.J.; Stenström, T.A. The Efficacy of Heat and Chlorine Treatment against Thermotolerant *Acanthamoebae* and *Legionellae*. *Scand. J. Infect. Dis.* **2004**, *36*, 656–662. [CrossRef] [PubMed]
40. Coulon, C.; Collignon, A.; McDonnell, G.; Thomas, V. Resistance of *Acanthamoeba* cysts to disinfection treatments used in health care settings. *J. Clin. Microbiol.* **2010**, *48*, 2689–2697. [CrossRef] [PubMed]
41. Dupuy, M.; Berne, F.; Herbelin, P.; Binet, M.; Berthelot, N.; Rodier, M.H.; Soreau, S.; Héchard, Y. Sensitivity of free-living amoeba trophozoites and cysts to water disinfectants. *Int. J. Hyg. Environ. Health* **2014**, *217*, 335–339. [CrossRef] [PubMed]
42. Erickson, M.C.; Ortega, Y.R. Review: Inactivation of protozoan parasites in food, water and environmental systems. *J. Food Prot.* **2006**, *69*, 2786–2808. [CrossRef] [PubMed]
43. Di Cesare, A.; Corno, G.; Manaia, C.M.; Rizzo, L. Impact of disinfection processes on bacterial community in urban wastewater: Should we rethink microbial assessment methods? *J. Environ. Chem. Eng.* **2020**, *8*, 104393. [CrossRef]
44. Giannakis, S.; Polo, M.I.; Spuhler, D.; Sánchez, J.A.; Fernández, P.; Pulgarin, C. Solar disinfection is an augmentable, in situ-generated photo-Fenton reaction—Part 1: A review of the mechanisms and the fundamental aspects of the process. *Appl. Catal. B Environ.* **2016**, *199*, 199–223. [CrossRef]
45. Adan, C.; Magnet, A.; Fenoy, S.; Pablos, C.; Águila, C.; Marugán, J. Concomitant inactivation of *Acanthamoeba* spp. and *Escherichia coli* using suspended and immobilized TiO_2. *Water Res.* **2018**, *144*, 512–521. [CrossRef] [PubMed]
46. Heaselgrave, W.; Kilvington, S. The efficacy of simulated solar disinfection (SODIS) against *Ascaris*, *Giardia*, *Acanthamoeba*, *Naegleria*, *Entamoeba* and *Cryptosporidium*. *Acta Trop.* **2011**, *119*, 138–143. [CrossRef]
47. Dupuy, M.; Mazoua, S.; Berne, F.; Bodet, C.; Garrec, N.; Herbelin, P.; Ménard-Szczebara, F.; Oberti, S.; Rodier, M.H.; Soreau, S.; et al. Efficiency of water disinfectants against *Legionella pneumophila* and *Acanthamoeba*. *Water Res.* **2011**, *45*, 1087–1094. [CrossRef] [PubMed]

48. Shu, L.; Zhang, B.; Queller, D.C.; Strassmann, J.E. *Burkholderia* bacteria use chemotaxis to find social amoeba *Dictyostelium discoideum* hosts. *ISME J.* **2018**, *12*, 1977–1993. [CrossRef] [PubMed]
49. Khan, N.A. Pathogenesis of *Acanthamoeba* infections. *Microb. Pathog.* **2003**, *34*, 277–285. [CrossRef] [PubMed]