

Article

Effect of a Commercial Disinfectant CLORICAN[®] on *Acanthamoeba* spp. and *Naegleria fowleri* Viability

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Abstract: Swimming pool water treatment by chemicals is an essential step to avoid microbial proliferation and infections namely caused by free living amoeba such as, for example, primary amoebic meningoencephalitis and *Acanthamoeba* keratitis. In the present study, a commercial reactive, CLORICAN, based on chlorine dioxide, was evaluated against *Acanthamoeba* spp. and *Naegleria fowleri*. We observed that CLORICAN could eliminate in a short period of incubation time both amoebae. Furthermore, *Naegleria fowleri*'s trophozoites were more sensitive than those of *Acanthamoeba* spp. By means of inverted microscopy, the chlorine dioxide was found to greatly affect morphology shape by increasing the cell size shrinkage.

Keywords: CLORICAN; *Acanthamoeba* spp.; *Naegleria fowleri*; amoebicidal effect



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1. Introduction

Free-living amoebae (FLA) are ubiquitous protozoa found in natural habitats, namely soil and water, and artificial habitats such as swimming pools. FLA act as pathogens, as parasites, as carriers, or as vehicles of pathogenic microorganisms such as bacteria or viruses [1]. At present, multiple species have been reported as being pathogens and opportunistic, namely *Acanthamoeba* spp., *Naegleria fowleri*, *Balamuthia mandrillaris*, *Vahlkampfia*, *Vermamoeba* and *Sappinia*. Based on the literature, *Acanthamoeba* spp., *Naegleria fowleri* and *Balamuthia mandrillaris* are the most common FLA, with severe pathogenesis. WHO have defined *Acanthamoeba* spp. and *Naegleria fowleri* as quality indicator microorganisms in recreational water [2].

Some *Acanthamoeba* species or genotypes are the causative agents of severe medical complications including pneumonitis, fatal granulomatous encephalitis, skin infections and *Acanthamoeba* keratitis (AK) [3–5]. Based on the small ribosomal subunit gene (18S rDNA), 22 different genotypes were identified as T1 to T22 [6]. Even though genotype T4 is the most abundant species in both clinical and environmental samples [7], other genotypes such as T2, T3, T5, T6, T11, T13, and T15 were isolated from AK and amoebic encephalitis cases [8]. *Acanthamoeba* are widely presented in freshwater and marine habitats such as rivers, tap water, swimming pools, and sea water, among others [9]. Overall, 25% of *Acanthamoeba* sp. isolated from the different habitats host and protect microorganisms from adverse environmental conditions and chemical factors [10,11]. Free living amoebae can be a reservoir of pathogenic bacteria such as *Vibrio cholerae*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Helicobacter pylori*, *Legionella pneumophila*, and *Klebsiella pneumoniae*,

among others. Virus such herpes virus, enteroviruses, pandoraviruses and mimivirus have also been isolated from FLA [12–14].

Nevertheless, the amoebae from the *Naegleria* genus were isolated particularly from fresh warm water such as hot springs, swimming pools, and lagoons, among others. Even though more than 40 species of *Naegleria* have been identified, only *Naegleria fowleri*, “the brain-eating amoeba”, has been reported to induce a rare and fatal disease, primary amoebic meningoencephalitis (PAM) [15,16].

In order to ensure the quality of recreational water such as swimming pools and so on, several water treatments are conducted, namely filtration, coagulation and disinfection [2]. Disinfection of recreational water is fundamental to eliminate any microbial hazards [17]. During this treatment, chemical agents (e.g., chlorination) and physical methods (e.g., UV-C radiation) are used in order to deactivate the present germs [2]. The CDC recommend a free chlorine concentration of at least 1 ppm in pools and at least 3 ppm in hot tubs/spas and a pH of 7.2–7.8 [18]. Chlorine dioxide has been reported as a strong disinfectant with numerous applications in wastewater treatment, environment and food disinfection, and medicine [19]. For instance, Dupuy et al. (2014) reported the efficacy of chlorine dioxide in inhibiting three different FLA strains [20]. In the present study, the efficacy of a new formulation based on chlorine dioxide was tested against four FLA strains—three *Acanthamoeba* strains and *Naegleria fowleri*. The protozoa were analyzed during the time using inverted microscopy.

2. Results

Figures 1–3 illustrates that a concentration above 0.063% of chlorine dioxide turned out to be lethal for *Acanthamoeba castellanii* Neff. In fact, at this concentration, the trophozoites seemed to lose any motility and appeared as fixed cells. After 24 h of incubation with chlorine dioxide at a concentration of 0.063% (Figure 2C), the trophozoites do not show their pseudopodia and become rounded cells. At the same concentration and after 96 h of incubation, we noticed that the rounded trophozoites were not able to encyst in order to remain as pseudo-cysts (Figure 3B). The same effect was observed for *Acanthamoeba polyphaga* and *Acanthamoeba griffini*, although both strains were more resistant to CLORICAN. As shown by Figure 4 for *Acanthamoeba griffini* and Figure 5 for *Acanthamoeba polyphaga*, a chlorine dioxide concentration of 0.126% was effective to prevent the encystation process. A concentration of 0.063% was able to eliminate 83% and 30%, respectively, of *Acanthamoeba castellanii* Neff and *Acanthamoeba griffini*. This dose was ineffective in *Acanthamoeba polyphaga*.

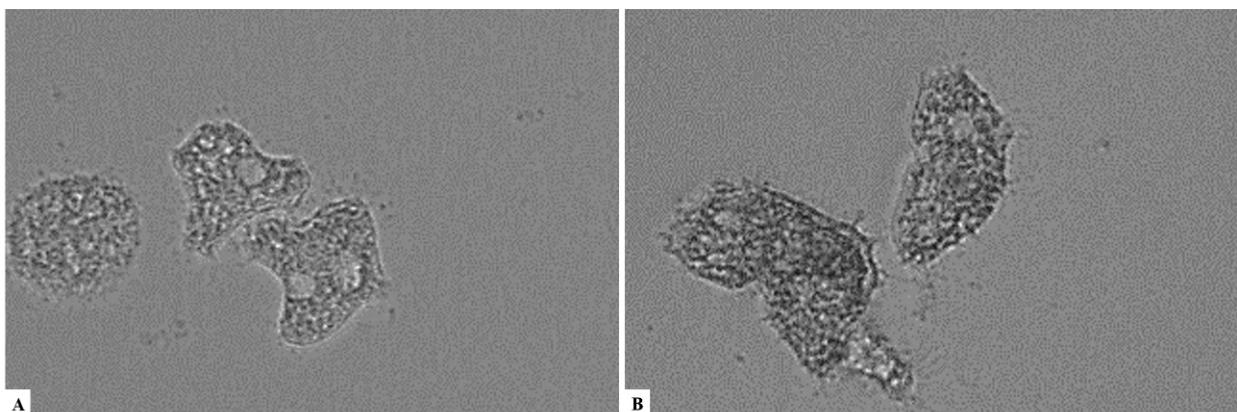


Figure 1. Cont.

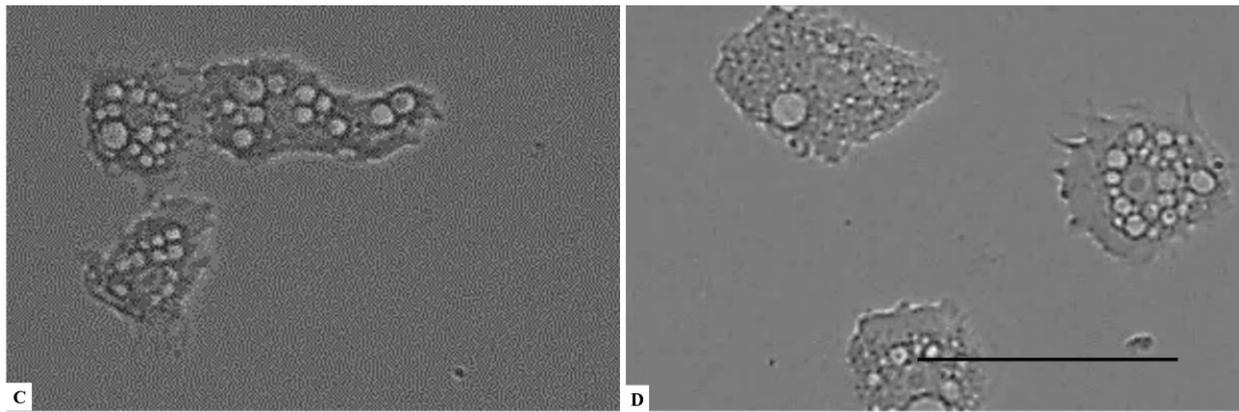


Figure 1. Effect of CLORICAN on *Acanthamoeba castellanii* Neff trophozoite after 3 h of incubation at 0.252% (A), 0.126% (B), 0.063% (C) of chlorine dioxide and negative control (D). At these concentrations, trophozoites seems to lose any motility and appeared as fixed cells (A,B). All images ($\times 40$) are representative of the population of treated amoebae and are based on the live cell imaging microscope EVOS FL cell imaging system. Scale bar = 50 μm .

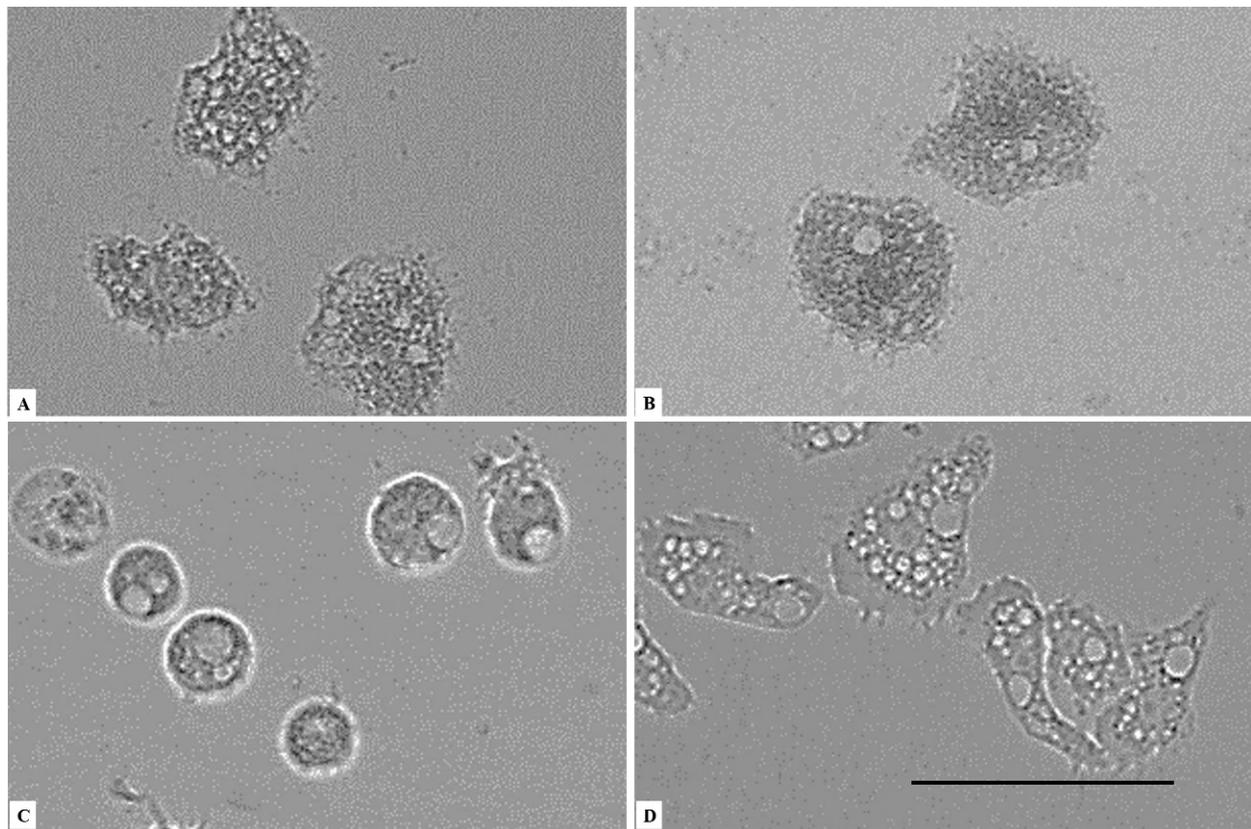


Figure 2. Effect of CLORICAN on *Acanthamoeba castellanii* Neff trophozoite after 24 h of incubation at concentrations of 0.252% (A), 0.126% (B), and 0.063% (C) chlorine dioxide, and the negative control (D). The characteristic pseudopodia of *Acanthamoeba castellanii* Neff cannot be observed in treated cells. (A–C) All images ($\times 40$) are representative of the population of treated amoebae and are based on the live cell imaging microscope EVOS FL cell imaging system. Scale bar = 50 μm .

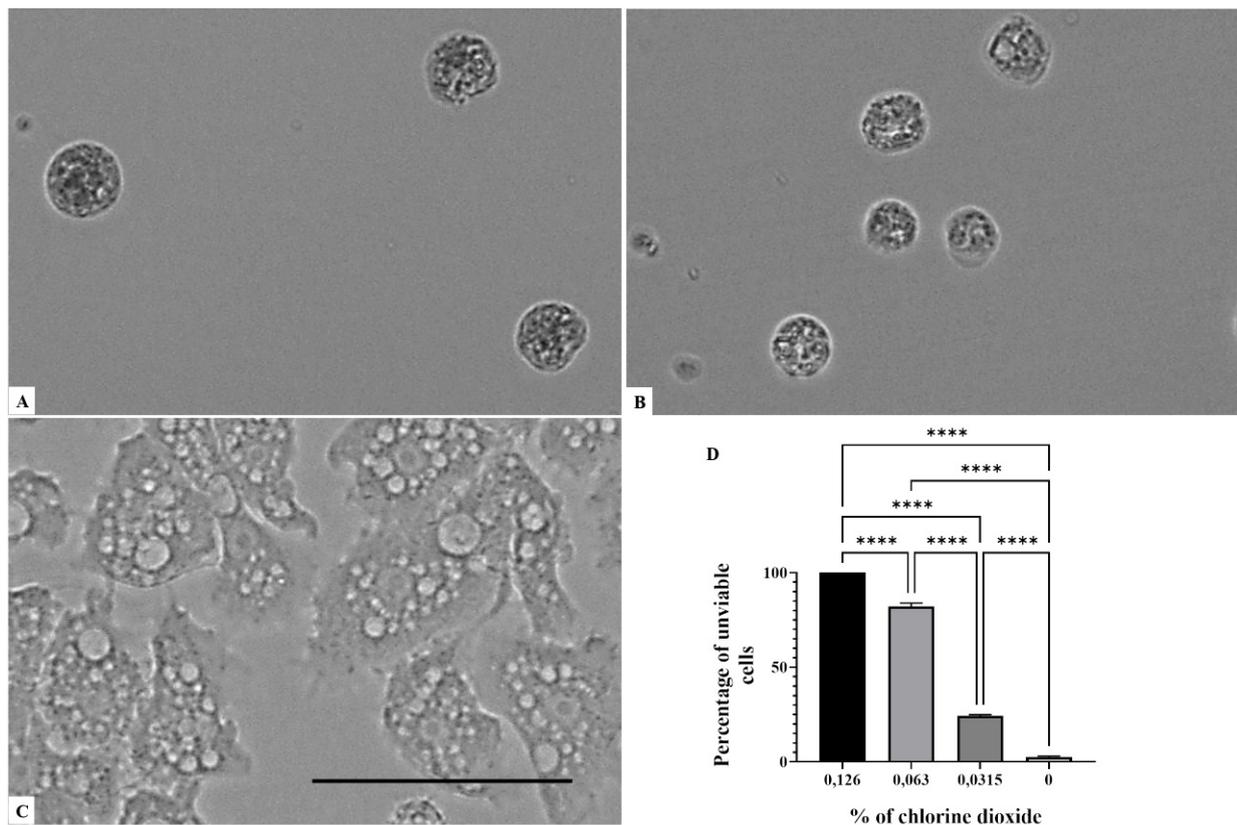


Figure 3. Growth inhibition of *Acanthamoeba castellanii* Neff after 96 h of incubation. *Acanthamoeba* spp. cells were incubated with a concentration of 0.126% chlorine dioxide (A). *Acanthamoeba* cells were incubated with a concentration of 0.063% chlorine dioxide (B). Negative control (C). Histograms of cells' unviability percentages for each tested concentration of chlorine dioxide (D). All images ($\times 40$) are representative of the population of treated amoebae and are based on the live cell imaging microscope EVOS FL cell imaging system. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ significance differences among different doses of chlorine dioxide. Scale bar = 50 μm .

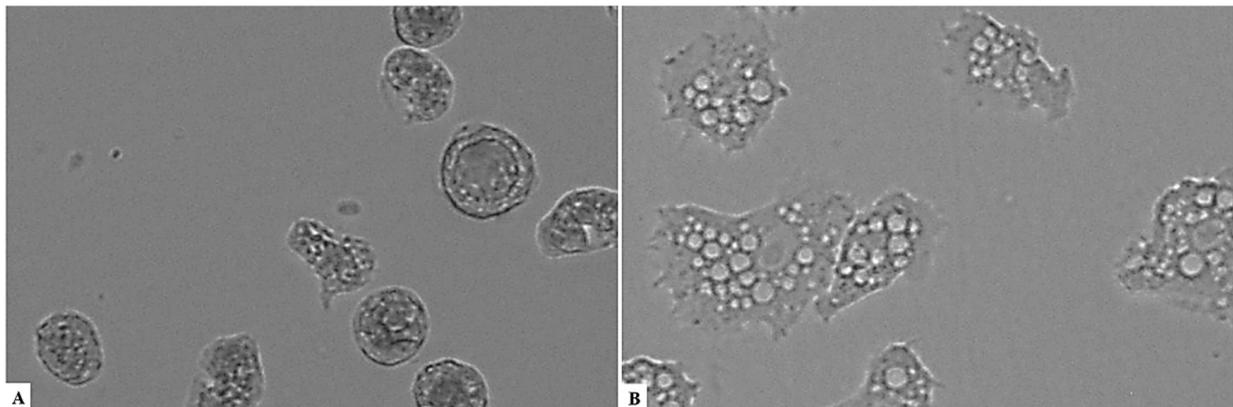


Figure 4. Cont.

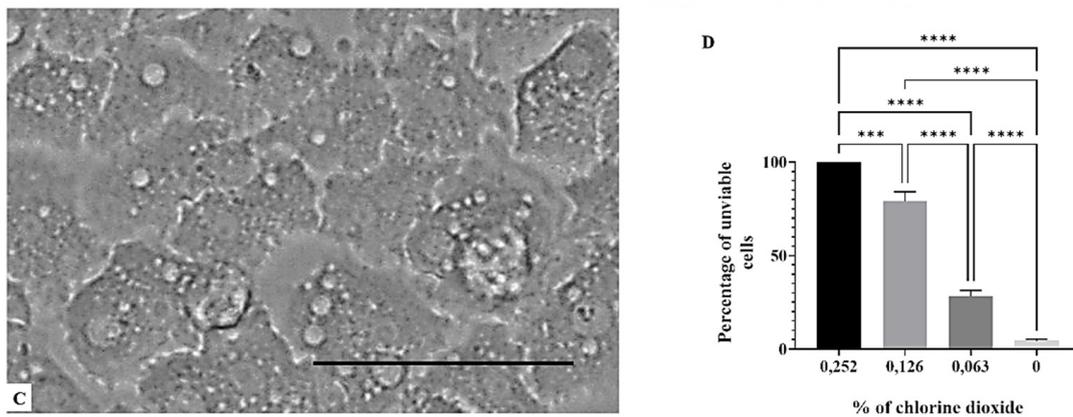


Figure 4. Growth inhibition of *Acanthamoeba griffini* after 96 h of incubation. *Acanthamoeba* spp. cells were incubated with a concentration of 0.126% chlorine dioxide (A). *Acanthamoeba* cells were incubated with a concentration of 0.063% chlorine dioxide (B). Negative control (C). Histograms of cells' unviability percentages for each tested concentration of chlorine dioxide (D). All images ($\times 40$) are representative of the population of treated amoebae and are based on the live cell imaging microscope EVOS FL cell imaging system. *** $p < 0.001$; **** $p < 0.0001$ significance differences among different doses of chlorine dioxide. Scale bar = 50 μm .

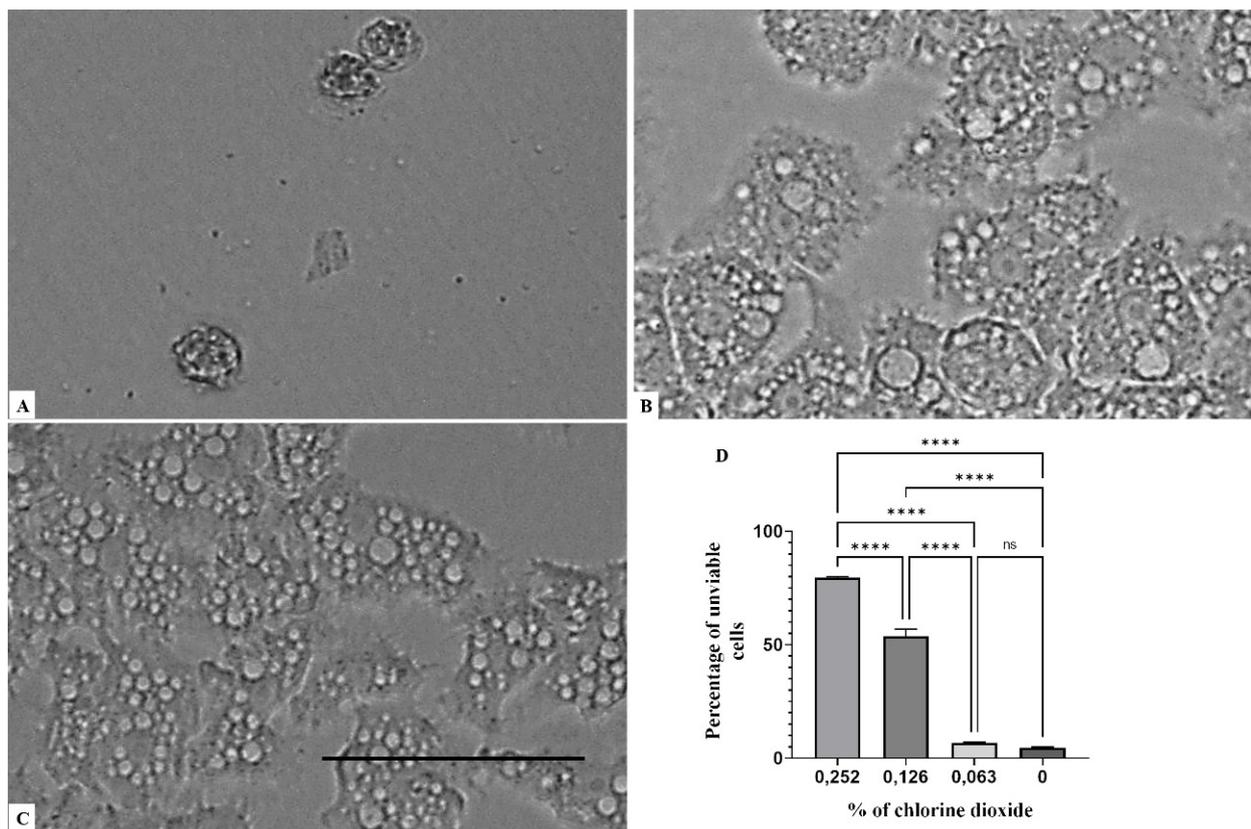


Figure 5. Growth inhibition of *Acanthamoeba polyphaga*; after 96 h of incubation. *Acanthamoeba* spp. cells were incubated with a concentration of 0.137% chlorine dioxide (A). *Acanthamoeba* cells were incubated with a concentration of 0.063% chlorine dioxide (B). Negative control (C). Histograms of cells' unviability percentages for each tested concentration of chlorine dioxide (D). All images ($\times 40$) are representative of the population of treated amoebae and are based on the live cell imaging microscope EVOS FL cell imaging system. Data are presented as means \pm SD (N = 3) **** $p < 0.0001$ significance differences among different doses of chlorine dioxide. Scale bar = 50 μm .

On the other hand, a chlorine dioxide concentration of up to 0.055 % on the *Naegleria fowleri* trophozoites, and a strong change in morphology was detected after 15 min of incubation; in fact, most of the amoebae lost their amoeboid shape, leading them to be rounded (Figure 6). This effect persists for at least 24 h (see Figures 7–9). At a lower concentration of chlorine dioxide, precisely 0.0275%, the trophozoite showed slight morphological changes as well as in the number of intracellular vesicles after 1 h of incubation (Figure 7). After 24 h of incubation with a 0.055% concentration of chlorine dioxide, we noticed the presence of pseudo-cyst cells; nevertheless, these cells are not able to complete the encystation process, as shown in Figure 9B.

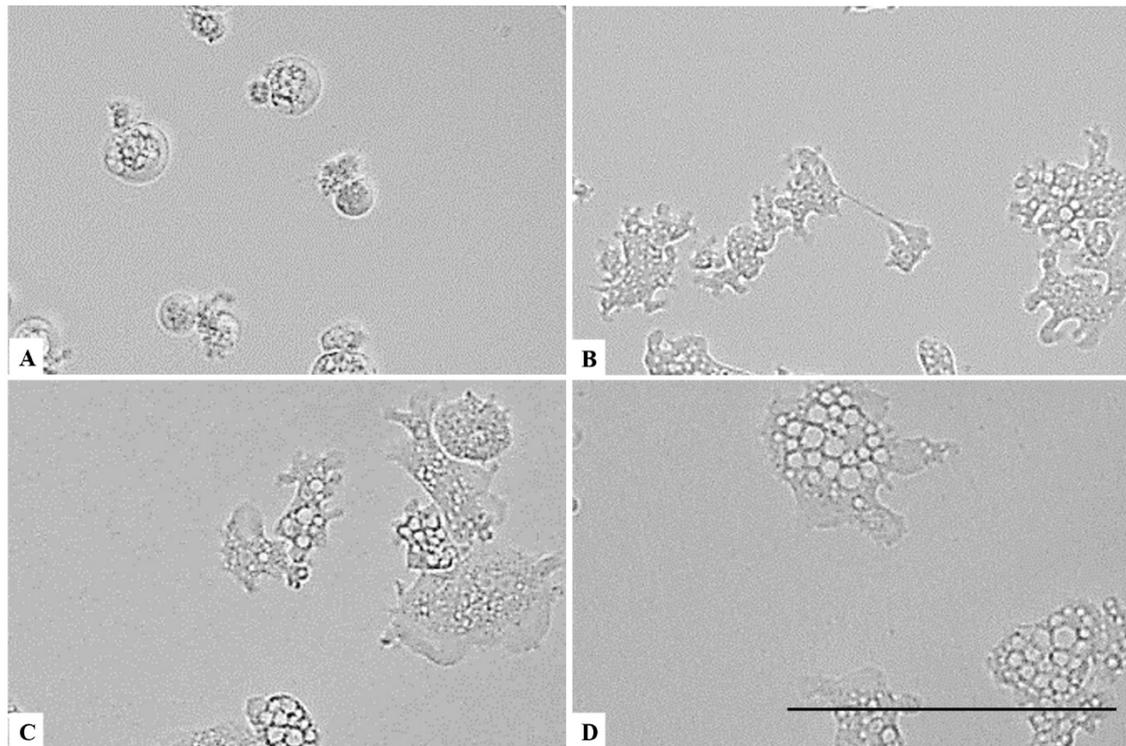


Figure 6. Effect of CLORICAN on *Naegleria fowleri* trophozoites after 15 min of incubation at chlorine dioxide concentrations of 0.11% (A), 0.055% (B), and 0.0275% (C) and the negative control (D). The *Naegleria fowleri* trophozoites lost their amoeboid morphology. All images ($\times 40$) are representative of the population of treated amoebae and are based on the live cell imaging microscope EVOS FL cell imaging system. Scale bar = 50 μ m.

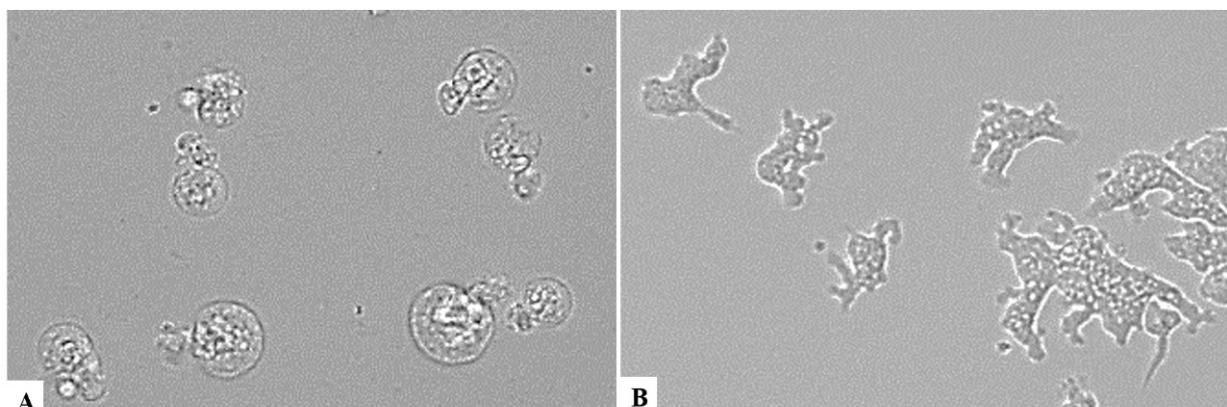


Figure 7. Cont.

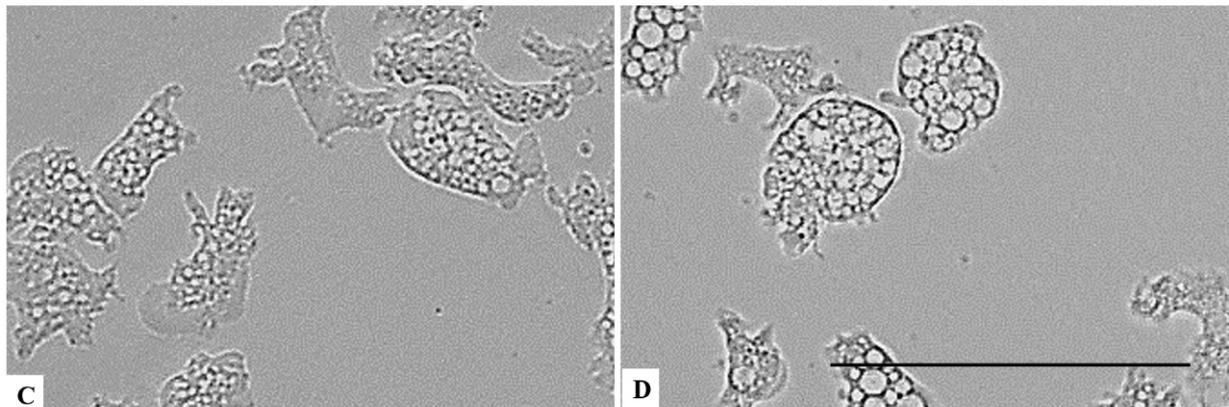


Figure 7. Effect of CLORICAN on *Naegleria fowleri* trophozoites after 1 h of incubation at chlorine dioxide concentrations of 0.11% (A), 0.055% (B), and 0.0275% (C) and the negative control (D). All images ($\times 40$) are representative of the population of treated amoebae and are based on the live cell imaging microscope EVOS FL cell imaging system. Scale bar = 50 μm .

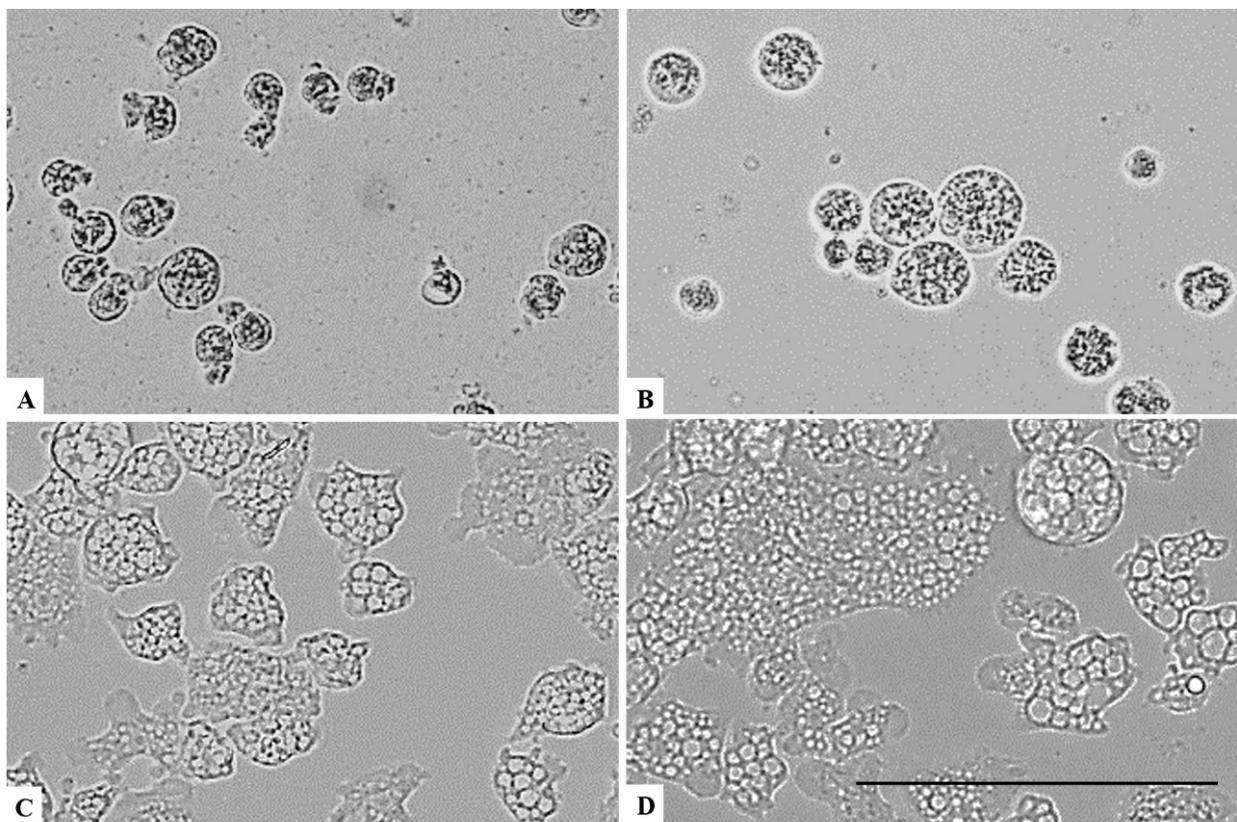


Figure 8. Effect of CLORICAN on *Naegleria fowleri* trophozoite after 24 h of incubation at chlorine dioxide concentrations of 0.11% (A), 0.055% (B), and 0.0275% (C) and the negative control (D). All images ($\times 40$) are representative of the population of treated amoebae and are based on the live cell imaging microscope EVOS FL cell imaging system. Scale bar = 50 μm .

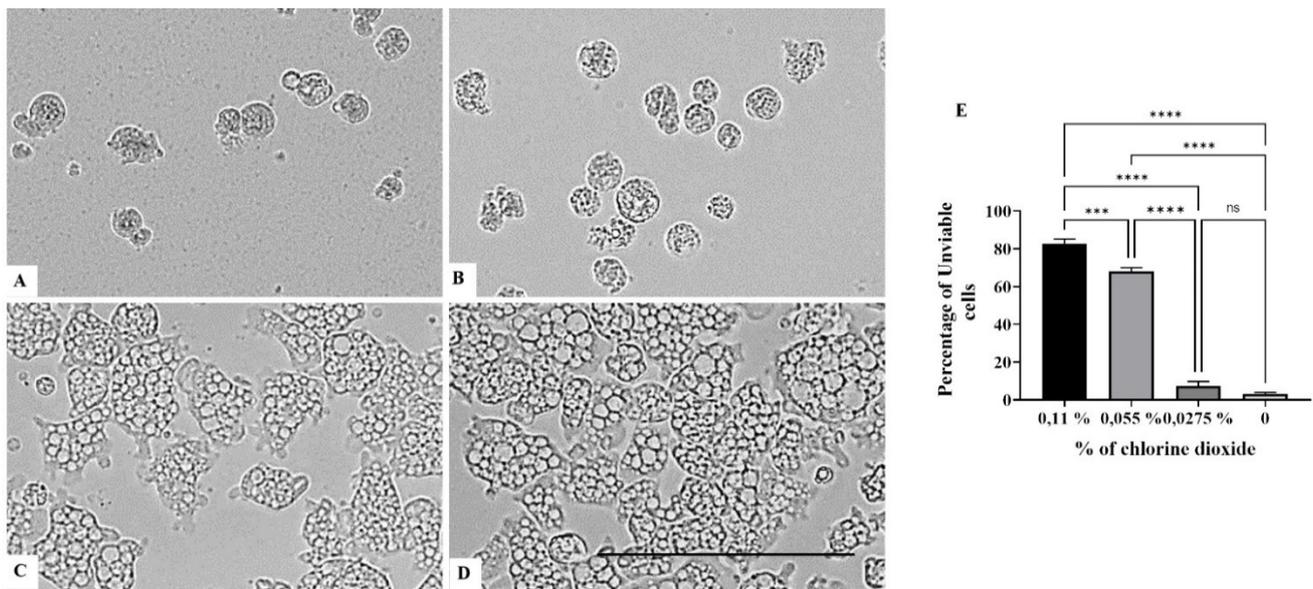


Figure 9. Effect of CLORICAN on *Naegleria fowleri* trophozoite after 48 h of incubation at chlorine dioxide concentrations of 0.11% (A), 0.055% (B), and 0.0275% (C) and the negative control (D). All images ($\times 40$) are representative of the population of treated amoebae and are based on the live cell imaging microscope EVOS FL cell imaging system. (E) Data are presented as means \pm SD (N = 3). *** $p < 0.001$; **** $p < 0.0001$ significance differences among different doses of chlorine dioxide. Scale bar = 50 μ m.

3. Discussion

Several free-living amoebae including *Naegleria fowleri* and some species from the *Acanthamoeba* genus represent a potential threat to human health due to their intrinsic pathogenicity and their role as protective and disseminating hosts for other pathogenic microorganisms [21]. Due to their higher resistance to diverse water treatments (physical and chemical), FLA may act as a Trojan horse transporting pathogenic bacteria such as *Legionella* spp., *Mycobacterium avium* and *Listeria monocytogenes*, among others [22]. Despite the fact that FLA have recently gained considerable attention due to their potential to develop illnesses, there is still a lack of information in methods to eliminate them [21]. Even though the disinfection treatment recommendations for recreational water are detailed by the WHO, CDC, and Commission for the Environment, Climate Change and Energy, among others, none of them include a specific protocol for free living amoeba eradication. The chemical disinfectants that are mostly used to eliminate pathogens are chlorine, chlorine dioxide and bromochlorodimethylhydantoin (BCDMH). In the WHO guideline, it is announced that the residual free chlorine level should not exceed 3 mg/L in public and semi-public pools and should not exceed 5 mg/l in public and semi-public hot tubs [2].

Chlorine dioxide, a potent oxidant and antimicrobial, is a disinfectant widely used in paper bleaching, surface disinfection, biofilm removal in water distribution systems, and, increasingly, in water treatment to produce potable water [18]. Several authors have reported the higher efficacy of chlorine dioxide than chlorine to eliminate free living amoeba cells [19]. Mogoa et al. (2010) reported that a concentration of free chlorine lower than 3 mg/L was not able to inhibit *Acanthamoeba castellanii* Neff, whereas in this study, a concentration of 3 mg/L was able to inhibit the growth of 60% of the cell culture [23]. On the other hand, the CDC recommends a concentration of 1–3 mg/L [18] of free chlorine to avoid the growth of *Naegleria fowleri* in swimming pools. Moreover, according to the Spanish recreational swimming pool regulation (RD 742/2013 27th of September), the recommended parametric value of free chlorine remains between 0.5 and 2 mg/L [24], while the CLORICAN product's indications establish a concentration of 0.4 mg/L for biocidal activity.

The amoebicidal capacity of CLORICAN on the trophozoite stage of *Acanthamoeba* spp. and *Naegleria fowleri* was studied over 48 h using an inverted light-microscope. In *Acanthamoeba castellanii* (ATCC 30234), Mogoia et al. (2010) reported that chlorine treatment led to the loss of pseudopodia and shrinking in the cell size and membrane permeabilization [23]. The same authors proved in a second paper that chlorine dioxide was as effective as chlorine in eliminating *Acanthamoeba castellanii* Neff by reducing the cell size more and inducing membrane permeabilization, as shown by propidium iodide staining [21].

In our study, CLORICAN was shown to have a stronger effect on *Naegleria fowleri* than *Acanthamoeba* spp. Several authors have mentioned the higher sensitivity of *Naegleria fowleri* to chlorine and chlorine dioxide compared to non-pathogenic *Naegleria*, namely *N. gruberi* and *Acanthamoeba* spp. [25]. The chlorine dioxide did affect *Naegleria fowleri* cells by altering the amoeboid shape and inducing cell shrinking.

4. Materials and Methods

4.1. Chemical

CLORICAN[®], a commercial liquid form of chlorine dioxide, is used to disinfect swimming pools. The reactive is produced by Vadeguas company, Canary Islands, Spain. The tested solution has a pH of 4.5–6 and a chlorine dioxide concentration of 0.75% (*w/w*).

4.2. Cell Culture

The strains used in this study were: *Acanthamoeba castellanii* Neff (ATCC 30010), *Acanthamoeba polyphaga*, genotype T4 (ATCC 30461) and *Acanthamoeba griffini*, genotype T3 obtained in previous studies [17]. Those strains were axenically grown in PYG medium (0.75% (*w/v*) proteose peptone, 0.75% (*w/v*) yeast extract and 1.5% (*w/v*) glucose) containing 40 µg/mL of gentamicin (Biochrom AG, Cultek, Granollers, Barcelona, Spain).

The *Naegleria* strain used was *Naegleria fowleri* (ATCC[®] 30808[™]). This strain was kept in the biological security facilities level 3 of our institution following Spanish biosafety guidelines for this pathogen. It was axenically cultured at 37 °C in 2% (*w/v*) bactocasitone medium (Thermo Fisher Scientific, Madrid, Spain) supplemented with 10% (*v/v*) fetal bovine serum (FBS), containing 0.5 mg/mL of streptomycin sulfate (Sigma-Aldrich, Madrid, Spain) and 0.3 µg/mL of penicillin G sodium salt (Sigma-Aldrich, Madrid, Spain).

4.3. In Vitro Activity Against Trophozoite Stage

The amoebicidal activity experiments were carried out using an inverted light-microscope Leica DM IL (Wetzlar, Germany). Initially, the trophozoites were counted using a Countess II FL automatic cell counter (Thermo Fisher Scientific, Madrid, Spain) to prepare a working concentration of 5.10^4 trophozoites/well and 10^5 cells/well, respectively, for *Acanthamoeba* spp. and *Naegleria fowleri* strains), and 50 µL per well was seeded in triplicate in a 96-well plate (Thermo Fisher Scientific, Madrid, Spain).

After that, a serial dilution of CLORICAN in the same culture medium was added to the plate (50 µL per well). As a negative control, the trophozoites were incubated with the medium alone. Plates were incubated with slight agitation for 96 h at 26 °C for *Acanthamoeba* spp. and 48 h at 37 °C for *Naegleria*. Subsequently, the plates were observed during the incubation time with an inverted light-microscope (15 min, 1 h, 24 h, 48 h and 96 h). The numbers of viable and nonviable trophozoite were counted using a Countess II FL automatic cell counter (Thermo Fisher Scientific, Madrid, Spain) at 96 h for *Acanthamoeba* spp. and 48 h for *Naegleria fowleri* using an inverted microscope and confirmed with trypan blue (0.4%) staining using the Countess II FL (Thermo Fisher Scientific, Madrid, Spain)

4.4. Statistical Analysis

All data are expressed as the mean ± standard deviation of at least three independent experiments. To highlight the chlorine dioxide's effect on *Acanthamoeba* spp., a statistical comparison was conducted using the *t*-test and one-way analysis of variance (ANOVA).

All the analysis and the graphics were done by GraphPad Prism version 8.0. Statistical significance was set at $p < 0.05$.

5. Conclusions

The CLORICAN reactive was highly effective at eliminating both amoeba strains tested in vitro. Moreover, *Naegleria fowleri*'s trophozoites were more sensitive than those of *Acanthamoeba* spp. The chlorine dioxide greatly affected the morphology shape and increased the cell size shrinkage. Further experiments are needed to better understand the mode of action of CLORICAN and to evaluate its in situ efficacy in a scaled-up process, e.g., in a swimming pool.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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