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CFDA-AM staining to assess the metabolic activity of *Giardia duodenalis* cysts inactivated by chlorine, boiling and ultraviolet irradiation

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ABSTRACT

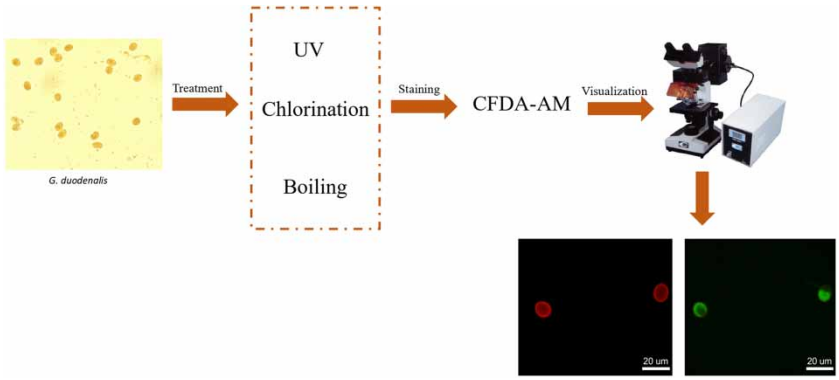
Giardia duodenalis is responsible for several waterborne gastrointestinal outbreaks worldwide. In addition to limitations presented by the main disinfection methods, assessing the inactivation efficiency of cysts after the treatment also poses challenges. Thus, this study aimed to use the 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) staining protocol to evaluate the viability of *G. duodenalis* cysts inactivated by different UV and chlorination doses and boiling times. Under epifluorescent microscopy, metabolically active cysts that presented green fluorescence were considered viable. In contrast, when no green fluorescence could be observed, organisms were considered non-viable. Although statistical analysis revealed that increasing the UV dose did not significantly decrease the percentage of viable cysts, the fluorescence signal intensity decreased considerably when the cysts were irradiated with a dose equal to or greater than 80 mJ cm^{-2}. Regarding chlorination and boiling treatments, this study demonstrated that no cyst showed fluorescence at the lowest NaClO concentration (0.5 mg/L) and in the shortest boiling time (2 min). Despite some limitations regarding the use of metabolic activity as a viability marker, this methodology is rapid, easy to run and cost-effective. Thus, we conclude that the CFDA-AM staining protocol has the potential to be used to assess *Giardia* cyst inactivation, although further research is required.

Key words: boiling, CFDA-AM, chlorination, *Giardia duodenalis*, ultraviolet irradiation, viability

HIGHLIGHTS

- 5-Carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) dye is efficient as a metabolic activity marker of *G. duodenalis* cysts.
- The CFDA-AM staining protocol is not the most indicated to assess cell viability after UV irradiation disinfection.
- No cyst showed metabolic activity at the lowest NaClO concentration and in the shortest boiling time.
- The CFDA-AM staining protocol is suitable to assess the inactivation of cysts in chlorination and boiling-based water treatment.

GRAPHICAL ABSTRACT



INTRODUCTION

Giardia duodenalis is an environmentally ubiquitous enteropathogen of humans and various other mammals (Thompson *et al.* 2005). This protozoan is responsible for 280 million diarrhea infections annually and causes nutritional imbalances and mortality, particularly in children and immunologically deficient people (Ankarklev *et al.* 2010; Thompson & Ash 2016).

Given the waterborne disease character of giardiasis, its etiological agent represents a public health risk in both developed and developing countries as it is responsible for numerous outbreaks in the world (Horton *et al.* 2019; Sammaro Silva & Sabogal-Paz 2021). Most outbreaks caused by *G. duodenalis* have been reported in the USA, but some cases have also been reported in other developed countries, such as Belgium and New Zealand (Efstratiou *et al.* 2017a). Concerning developing countries, although no Giardiasis waterborne outbreaks have been documented in Latin America in recent years, *Giardia* spp. along with *Cryptosporidium* spp. have been the most frequently protozoa found in water samples (Rosado-García *et al.* 2017).

G. duodenalis has a simple life cycle with two main stages, the proliferating trophozoite which multiply by binary division in the small intestine lumen and the infectious cyst that is responsible for the parasite surveillance in the environment and is usually excreted in high numbers in the feces of infected hosts (Rousseau *et al.* 2018). Thus, transmission occurs mainly through fecal–oral routes, with sources including other infected humans or animals, contaminated water and food (Cacció & Lalle 2015).

In addition to being very resilient to environmental conditions, *Giardia* cysts are also resistant to chemical disinfection, which become a challenge for health agencies as chlorination has been used as a disinfectant in water and wastewater treatment worldwide (Adeyemo *et al.* 2019). Moreover, disinfection of household drinking water with free chlorine is largely used in developing countries because it is effective, available, easy to dose properly and inexpensive (WHO 2011). However, due the resistance of *Giardia* to chlorine, alternative disinfectants such as ultraviolet (UV) light have been suggested and are used in many countries.

UV irradiation is a treatment that has attracted increasing attention for use in drinking water and sewage systems worldwide and involves genetic damage resulting in the disturbance and/or inhibition of cell division, growth and reproduction (Delacroix *et al.* 2013). However, some limitations of UV use can be found in developing countries, especially the dependency on constant and secure power supply which is not mostly available in many parts of developing countries (Vilhunen *et al.* 2009).

Another widely used water disinfection treatment is boiling. Although the disadvantages of boiling include no reduction in sediment or turbidity and the fact that boiling may not be a cost-effective or practical option in many places, this heat-based technology remains the most common form of household-scale water treatment worldwide, having been used to treat drinking water since ancient times (WHO 2011).

In addition to the limitations presented by each disinfection method, assessing the inactivation efficiency of the cysts after the treatment also poses challenges. The gold standard analysis to evaluate protozoa inactivation is animal infectivity (Sammaro Silva & Sabogal-Paz 2020). However, this technique is very expensive and may take a long time to provide conclusive data (Santos *et al.* 2015). A widely used alternative, especially in the environmental context, is to adopt viability assays, which is an inference about the amount of living cells based on their cellular integrity or metabolic activity. This methodology does not require animal use and is, therefore, less labor-intensive and costly (Sammaro Silva & Sabogal-Paz 2020).

Most of the studies that investigate *Giardia* spp. cyst viability uses dye-exclusion approaches. These methods are based on dyes that can penetrate selectively into cells that have lost their membrane integrity and are excluded by live cells; thus, these dyes stain parasites that are dead (Sammaro Silva & Sabogal-Paz 2020). Dyes that have been used successfully for this assessment include non-fluorescent ones, such as trypan blue, and fluorescent ones, such as propidium iodide (PI). However, a major limitation of dye-exclusion approaches is that a cell can have an intact membrane but will nevertheless be non-viable. Thus, parasites that are non-viable but have an intact membrane will not stain with those dyes. Hence, the dye-exclusion technique leads to the overestimation of exposure of humans to infective parasites and to the underestimation of the efficacy of control measures (Rousseau *et al.* 2018).

In an attempt to overcome some of these limitations, methods based on diffusing specific dyes into living cells have been developed (Rousseau *et al.* 2018). One example is the non-fluorescent 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM). This dye diffuses through the cell membrane and then, following cleavage by intracellular

enzymes present only in viable cells, a green, fluorescent molecule is produced and retained in cells with intact membranes (Olsen *et al.* 2015, 2016).

CFDA-AM has been successfully used in studies on phytoplankton (Gorokhova *et al.* 2012; Olsen *et al.* 2015, 2016). However, it has never been used to discriminate dead from live cysts of *G. duodenalis*. Thus, the aim of the present study was to use the CFDA-AM staining protocol to evaluate the viability of *G. duodenalis* cysts inactivated by three different disinfection methods such as UV irradiation, chlorination, and boiling.

MATERIALS AND METHODS

G. duodenalis samples

The study was approved by the Ethics Committee in Animal Research at the University of São Paulo (CEUA/EESC-USP) under protocol N° 02/2021.

Experiments were performed utilizing contaminated feces donated by the Municipal Kennel of São Carlos, São Paulo, Brazil. *G. duodenalis* cysts were purified using the zinc sulfate centrifugal flotation technique (Faust *et al.* 1938). Slides and coverslips with positive samples were washed with sodium phosphate-buffered saline (PBS), pH 7.2, and then transferred to polystyrene microtubes. They were submitted to three centrifugations at 10,000 × *g* for 10 min each. In each centrifugation, the supernatant was discarded, and a new PBS was added. The final pellet was resuspended in 500 μL of PBS and stored between 4 and 8 °C until used. Experiments on cysts were initiated within 48 h from the time the cysts were shed. To avoid possible interference with the viability testing, no chemicals or preservatives were added to the cyst suspensions.

Treatments

Approximately 300 *G. duodenalis* cysts (297 ± 77 cysts) were submitted to three types of inactivated treatments, UV irradiation, chlorination and boiling. All experiments were conducted in triplicate.

For the UV irradiation treatment, the cysts were suspended in 100 μL of Hank's Balanced Salt Solution (HBSS), placed in transparent plastic taps (15-mm diameters) and exposed to a low pressure UV lamp with an output at 254 nm using a collimator at room temperature (RT). The intensity of the UV light, measured using a digital radiometer, was 350 μW/cm^2 at 254 nm. A rig was set up 10 cm below the lamp, and a position where the UV intensity was maximal was marked. According to Campbell & Wallis (2002), UV irradiation at 20–40 mJ cm^{-2} can kill 99.9% of *Giardia* cysts (3 log). In the present study, the UV doses applied were the following 10, 20, 40, 80, 160 and 320 mJ cm^{-2}, which represent exposure times of 30, 63, 126, 252, 505 and 1.012 s, respectively.

Regarding chlorination treatment, doses ranging from 0 to 5 mg/L (0, 0.5, 1, 2, 3, 4 and 5) of sodium hypochlorite (NaClO) were tested, following WHO recommendations (WHO 2011). In 0.6 mL microtubes, the chlorine diluted in distilled water was added to the suspended cysts and kept in the dark for adsorption. After 30 min of contact time, the chlorine reaction was neutralized with sodium metabisulphite ($Na_2S_2O_5$) at a concentration ratio of $Na_2S_2O_3$ to chlorine of 3:1 (Qin *et al.* 2014) in a final volume of 100 μL.

According to the WHO Guidelines for Drinking-water Quality, the process of heating water to a rolling boil is sufficient to inactivate pathogenic bacteria, viruses and protozoa, including *Giardia* cysts (WHO 2017). For this study, cysts were suspended in 100 μL of HBSS in 0.6 mL microtubes and boiled at 100 °C using a water bath for 2, 5, 10 and 15 min.

Staining

After each treatment, *G. duodenalis* cysts were kept in 0.6 mL microtubes and stained with the esterase substrate CFDA-AM (C1354, Invitrogen Inc., Carlsbad, CA, USA) prior to epifluorescent microscopy according to the protocols described by Olsen *et al.* (2015). Aliquots of 10 mM CFDA-AM in dimethyl sulfoxide (DMSO) were kept at −20 °C. About 1 mM work solution was prepared fresh each day by diluting it with Milli-Q water. Samples were stained to a final concentration of 2 μM, followed by incubation for 60 min at RT at dark conditions. Then, wall cysts were labeled using a red-labeled anti-*Giardia* monoclonal antibody (Giardia-a-Glo, 1:20 optimal dilution, Waterborne, Inc.) for 30 min at 37 °C in the absence of direct light.

Control samples included were (1) untreated (non-irradiated, non-chlorinated and non-boiled) cells both unstained and dual stained and (2) dead cells killed using 5% formalin followed by the staining procedure. The dead stained cells were not expected to fluoresce green, but red.

Microscopy analysis

After staining, the 100 μL samples of each treatment were divided into two slides (50 μL each) and examined using an immunofluorescence microscope (Olympus® BX51). The cysts were identified under a 500-nm excitation and 630-nm emission filter as bright red organisms. Then, metabolic activity was assessed under the maximum excitation of 495 and 520 nm emission filters, compatible with the carboxyfluoresce product obtained from CFDA-AM hydrolysis. Metabolically active cysts were expected to present green fluorescence and were considered viable. Organisms were considered dead (non-viable) when no green fluorescence could be observed. The enumeration of viable and not viable cysts was performed at 400× magnification. Photography was performed using Image-Pro® 6.3 software and a digital camera attached to the microscope.

Statistical analysis

Normality tests were performed to determine dataset distribution and the most suitable type of statistical test. As all datasets were normally distributed (Shapiro–Wilk test, $p > 0.05$), the *T*-test and the several-samples ANOVA test were applied to compare the samples and determine if there was a significant difference between them. All statistical analyses were performed on PAST 3.25 software (PAlaeontological STatistics) considering a significance level of 5%.

RESULTS

Microscopic evaluation of CFDA-AM-stained *G. duodenalis* cysts

G. duodenalis cysts exposed to disinfection methods were stained with CFDA-AM to assess metabolic activity. Additionally, wall cysts were labeled using a red-labeled anti-*Giardia* monoclonal antibody. As a result, metabolically active cysts display red fluorescence, as well as green fluorescence from internal cellular structures, as can be seen in non-treated samples ((Figure 1(a)–1(d)). On the other hand, inactivated cells displayed red fluorescence but no internal green fluorescence, indicating loss of viability (Figure 1(e)–1(f)).

G. duodenalis cyst inactivation treated with chlorine, boiling and UV irradiation

In order to assess metabolic activity, CFDA-AM-stained *G. duodenalis* cysts were submitted to three different disinfection methods: UV irradiation (10–320 mJ cm^{-2}), chlorination (0–5 mg L^{-1} for 30 min) and boiling (2–15 min at 100 °C).

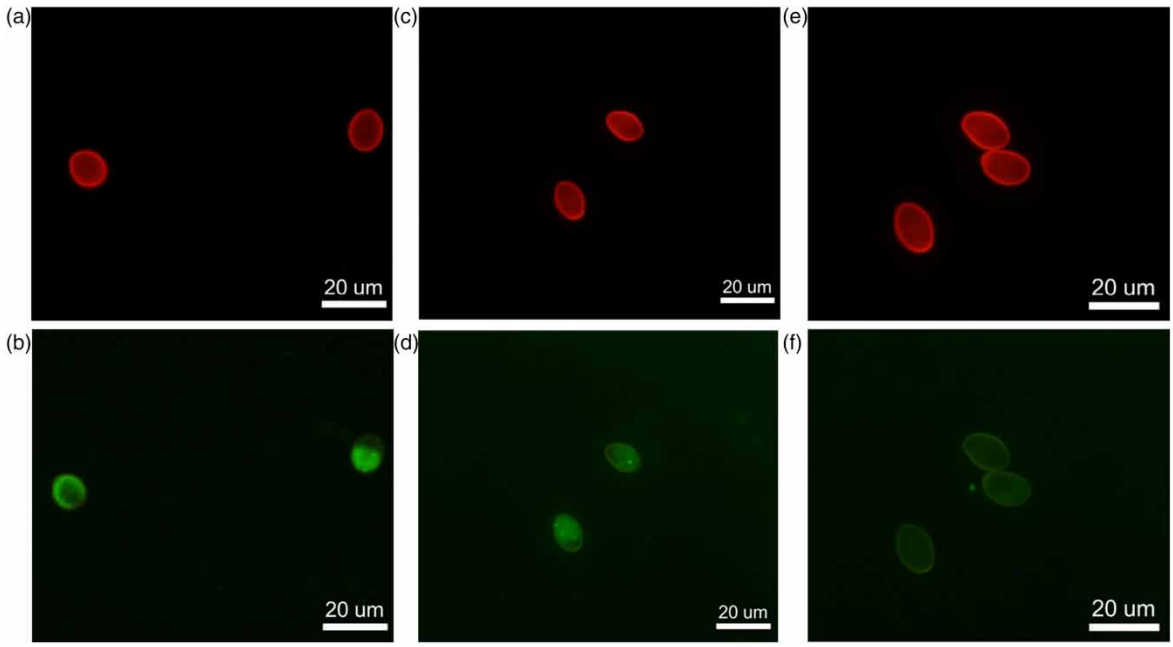


Figure 1 | Epifluorescent microscopy of CFDA-AM-stained *G. duodenalis* cysts. Pictures (a–d) show metabolically active (viable) cysts with red (a and c) and internal green fluorescence (b and d). Pictures (e and f) show metabolically inactive (unviable) cysts with red fluorescence (e) and no internal green fluorescence (f). Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wh.2022.092.

The inactivation efficiency results for each disinfection method are shown in Figure 2. Disinfection by chlorine and boiling is presented in only one experimental condition each (15 mg min L^{-1} of NaOCl and 2 min of boiling), while disinfection by UV irradiation is in several ones. From 15 mg min L^{-1} of NaOCl and 2 min of boiling, all *G. duodenalis* cysts were considered non-viable as no internal green fluorescence was observed in these samples after treatment, and the disinfection efficiencies were >2.46 $\log_{10}$ (maximum inactivation value). Regarding the UV irradiation technique, all samples still have viable cysts and the maximum inactivation of 0.54, 0.50 and 0.48 $\log_{10}$ were reached in doses of 40, 160 and 320 mJ cm^{-2}, respectively. Statistical analyses demonstrated that the UV irradiation did not demonstrate significant changes in the viability of irradiated compared to non-irradiated cysts ($p = 0.74$, several-samples ANOVA). Besides, the chlorination and boiling technique efficiencies were not statistically different from each other to assess the viability of CFDA-AM-stained *G. duodenalis* cysts ($p = 0.92$, *T*-test), but different from the UV irradiation technique ($p = 0.60$, several-samples ANOVA).

Other information about the disinfection technique by UV irradiation was extracted, such as the curve of viable cyst percentage at different UV doses and its adjustment to standard mathematical models (Figure 3). Correlation analysis of experimental and theoretical data indicated a high correlation and a trend of an exponential decrease of viable CFDA-AM-stained *G. duodenalis* cysts with the increasing of UV light doses ($r^2 = 0.8978$). However, even with a decay trend, statistical analyses did not indicate a difference in the viability percentage between the disinfected samples ($p = 0.53$, several-samples ANOVA), although the intensity of the fluorescence signals decreased considerably when the cysts were irradiated with a dose equal to or greater than 80 mJ cm^{-2} (Figure 4).

DISCUSSION

G. duodenalis is a widespread enteropathogen that poses a challenge to public health as it is resistant to conventional disinfection methods (Sammaro Silva & Sabogal-Paz 2021). Thereby, when cysts of this parasite are targets, it is challenging to infer inactivation efficacy, as it may require labor-intense and costly methodologies (Santos *et al.* 2015). In order to overcome these limitations, fluorescent dye-based methods have been commonly used as colored markers of viability, especially by laboratories that lack infrastructure for molecular examination or infectivity tests (Sammaro Silva & Sabogal-Paz 2020).

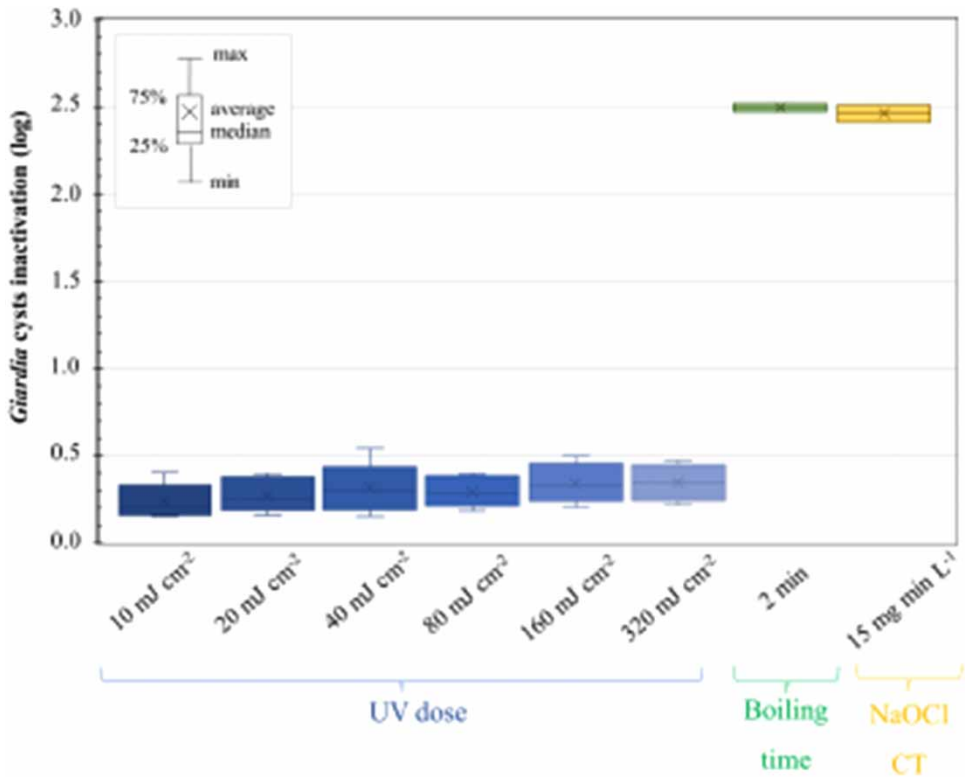


Figure 2 | CFDA-AM-stained *G. duodenalis* cyst inactivation after the treatment with chlorine, boiling and UV irradiation.

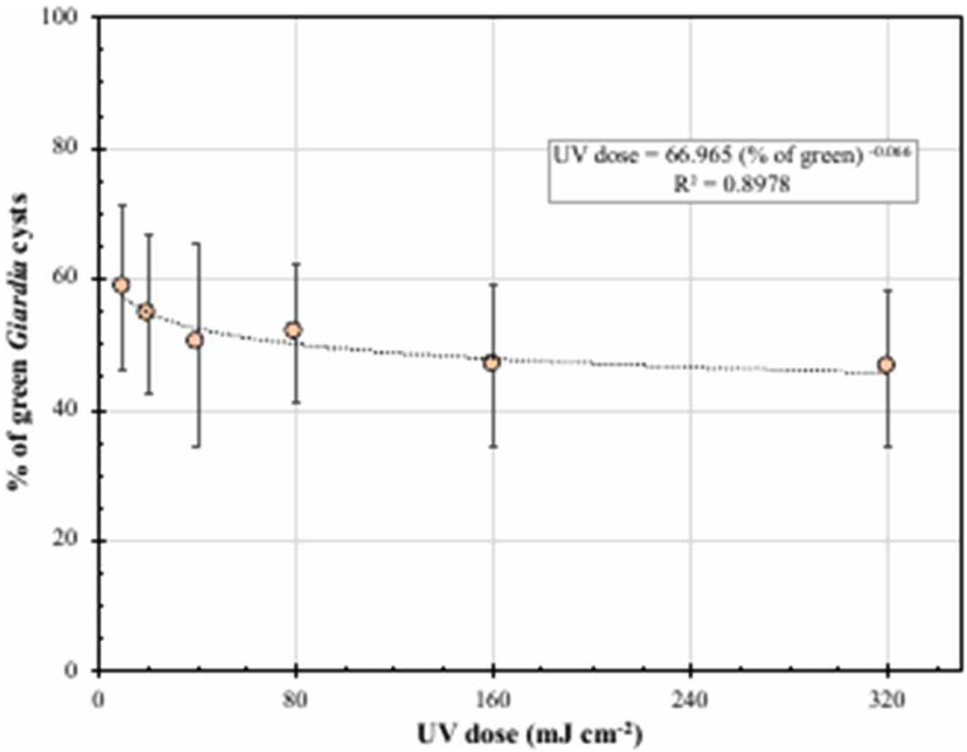


Figure 3 | Percentage of viable CFDA-AM-stained *G. duodenalis* cysts after the treatment with UV doses ranging from 10 to 320 mJ cm^{-2}.

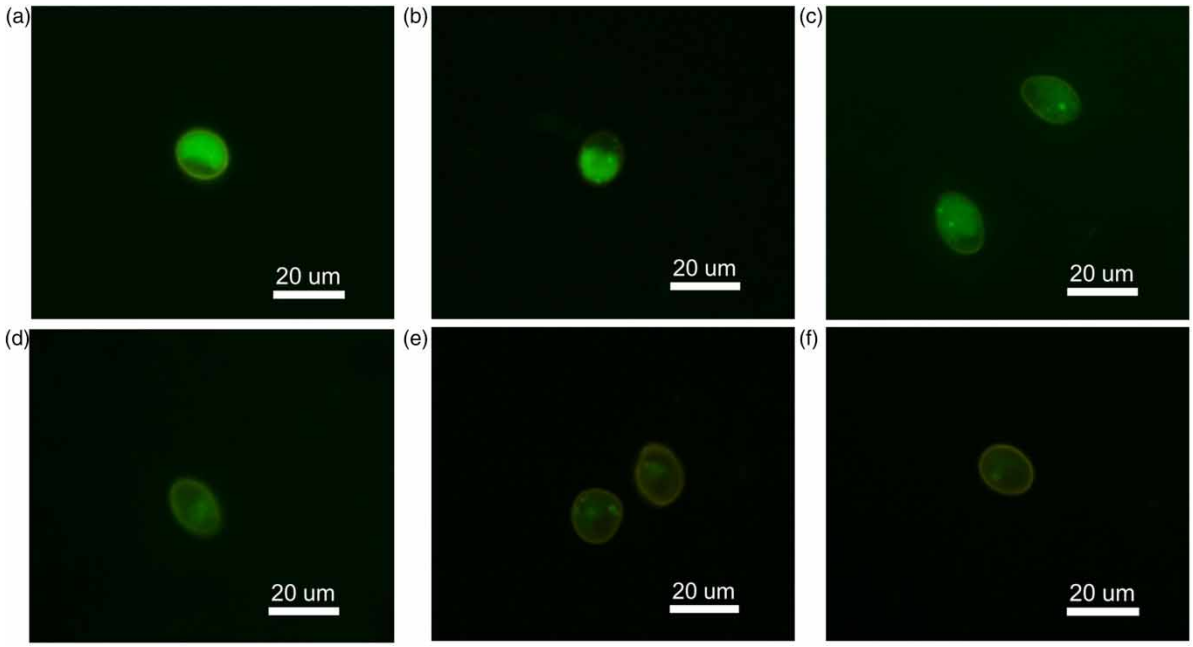


Figure 4 | Epifluorescent microscopy of CFDA-AM-stained *G. duodenalis* cysts treated with UV irradiation. The intensity of the fluorescence signals decreased considerably when cysts were irradiated with 80, 160 and 320 mJ cm^{-2} (d–f, respectively), compared to the cysts irradiated with 10, 20 and 40 mJ cm^{-2} (a–c, respectively).

In this study, we used the metabolic activity (intracellular hydrolysis) of *G. duodenalis* cysts as an indicator of viability. When incubated with the CFDA-AM substrate, cells that displayed red fluorescence, as well as green fluorescence from internal cellular structures, were considered viable and could easily be distinguished from dead cells, displaying red fluorescence only. Although this is the first report of using CFDA-AM to assess the metabolic activity of *G. duodenalis* cysts, our results support previous studies that have used this dye for microscopically evaluation of live/dead detection of *Tetraselmis suecica* (Gorokhova *et al.* 2012; Olsen *et al.* 2015, 2016).

In the present study, *G. duodenalis* cysts were inactivated with UV irradiation, chlorination and boiling and then stained with CFDA-AM for viability assessment.

Considering UV treatment, previous studies have shown that irradiation at 10–40 mJ cm^{-2} is capable of killing 3 log of *G. duodenalis* cysts (Campbell & Wallis 2002; Einarsson *et al.* 2015). However, our results failed to demonstrate significant changes in the viability of irradiated compared to non-irradiated cysts. The difference in results may be related to the technique applied to verify the viability of the cysts. In the previous studies, the viability of cells after UV treatment was measured by excystation or animal infectivity, while the present work used a metabolic activity indicator dye.

In fact, former studies have indicated that Mongolian gerbils (*Meriones unguiculatus*) are the most efficient model used to distinguish viable from non-viable cysts (Rousseau *et al.* 2018). However, in addition to it being costly for routine examinations and raising ethical concerns, *in vivo* methods are time-consuming (Sammaro Silva & Sabogal-Paz 2020). Thus, a rapid protocol such as CFDA-AM staining is of interest, especially in the context of environmental engineering and sanitation that requires earlier decision-making.

It is also important to highlight that UV irradiation does not always cause immediate inactivation of all cells but can cause DNA damage that inhibits normal transcription and replication. Thus, the cells are in an unculturable state, although still with esterase activity (Davey 2011; Hammes *et al.* 2011).

Although statistical analysis revealed that increasing the UV dose did not significantly decrease the percentage of viable cysts, the intensity of the fluorescence signals decreased considerably when the cysts were irradiated with a dose equal to or greater than 80 mJ cm^{-2}. The fluorescence intensity from esterase substrates is correlated with the metabolic activity in the cell, and hence the fluorescing signal will vary over a large range of intensities depending on the level of this activity (Brookes *et al.* 2000; Olsen *et al.* 2015). Therefore, the decrease in fluorescence intensity observed in cysts subjected to UV dose equal to or greater than 80 mJ cm^{-2} probably indicates a change in viability, specifically caused by UV damage (Olsen *et al.* 2015). These results are extremely relevant, especially in the environmental area, where assessing the viability of protozoa after the treatment still poses challenges. Thus, although further studies are required, the CFDA-AM staining protocol shows to be a rapid and cost-effective technique and has the potential to assess the viability of *Giardia* cysts inactivated by UV irradiation, a treatment that has been widely used in recent years.

Regarding chlorination and boiling treatments, this study demonstrated that no cyst showed green fluorescence at the lowest NaClO concentration (0.5 mg/L) and in the shortest boiling time (2 min). Probably, the cell membrane integrity was strongly compromised by chlorine and heating. According to Gorokhova *et al.* (2012), only an intact membrane can maintain the cytoplasmic milieu which is needed to support esterase activity. Thus, the absence of esterase activity found in this study may indicate that chlorine and boiling cause immediate cell inactivation.

The results obtained herein regarding chlorination corroborates a recent study that has demonstrated that *Giardia* cysts that are present in wastewater samples and treated with a low dose of chlorine (0.5 mg/L) for 30 min became non-viable (Adeyemo *et al.* 2019). Therefore, it can be concluded that when the CFDA-AM staining protocol is used as a metabolic activity indicator, increasing the dose of hypochlorite beyond 0.5 mg/L makes no difference to *Giardia* viability. Moreover, using high doses of chlorine for the sole purpose of inactivate *Giardia* in water and wastewater treatment plants may lead to a waste of resources (Adeyemo *et al.* 2019).

Finally, data related to inactivation temperature of *Giardia* cysts are limited. To the best of our knowledge, there are no studies in the literature that have examined *Giardia* thermal inactivation at temperatures approaching 100 °C. However, according to the WHO Guideline, inactivation at temperatures ranging from 50 to 70 °C has been reported (WHO 2015). Furthermore, our results support the WHO recommendation of heating water to a rolling boil to inactivate pathogenic bacteria, viruses and protozoa in cases of household-scale water treatment (WHO 2017).

It is important to emphasize that due to limitations in cyst supply, this study utilized a small volume (100 μL) of *G. duodenalis* suspension, which may have favored inactivation in a low NaClO concentration and shortest boiling

time. Thus, further studies with greater volume of working solutions are recommended to simulate the real volume utilized in water treatment plants and in household-scale water treatment.

It is also important to notice that in this study, CFDA-AM was used as a marker of enzymatic activity. Thus, cysts showing any green fluorescence intensity were considered viable and those without green fluorescence were related to loss of enzymatic activity as might be expected of inactive and potentially dead cells. However, the use of esterase activity as a viability marker should be treated with caution as some assays have demonstrated that enzyme activity does not always indicate cell viability since there are several cases in which dead cells show esterase activity, probably due to facilitated stain transport into the cell (Santos *et al.* 2015).

CONCLUSIONS

Metabolic activity (intracellular hydrolysis) of *G. duodenalis* cysts was used as an indicator of viability in this study. The CFDA-AM staining protocol was efficient in demonstrating significant changes in the viability of chlorinated and boiled cysts compared to the non-treated ones but failed in the UV-treatment assays. Despite the limitations regarding the use of esterase activity as a viability marker, this methodology is rapid, easy to run and cost-effective (nearly $3.29 per assay – exchange rate of January 3, 2022). Thus, we conclude that the CFDA-AM staining protocol has the potential to assess the inactivation of *Giardia* cysts and to be applied in water studies that aim to assess the risk for consumption or reuse; however, more research is required.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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