Analytical challenges and perspectives of assessing viability of *Giardia muris* cysts and *Cryptosporidium parvum* oocysts by live/dead simultaneous staining

Kamila Jessie Sammarro Silva, Lyda Patricia Sabogal-Paz*

Department of Hydraulics and Sanitation, São Carlos School of Engineering, University of São Paulo, Avenida Trabalhador São-carlense 400, São Carlos, São Paulo 13566-590, Brazil.

E-mail: lysaboga@sc.usp.br

Orcid: Kamila Jessie Sammarro Silva https://orcid.org/0000-0002-6881-4217

Lyda Patricia Sabogal-Paz http://orcid.org/0000-0003-2753-3248

Abstract

Giardia and *Cryptosporidium* are pathogenic protozoa often present in the environment in their infective form (cysts and oocysts). These parasites are very resistant to disinfection, which makes them important target organisms in environmental quality monitoring and sanitation. Viability assessment provides an interpretation of cell inactivation, and it can be evaluated by membrane integrity as well as enzyme activity, using different staining methods. These are straightforward and adequate to laboratories that lack infrastructure for molecular-based technologies or animal infectivity tests. This study investigated simultaneous staining by a commercial live/dead kit, in order to assess viability of *Cryptosporidium parvum* oocysts and *Giardia muris* cysts, comparing it to propidium iodide (PI) incorporation, a common stain applied in viability estimation. Results suggested that, although the central hypothesis of one-panel visualization (α=0.05) was met, simultaneous staining impaired (oo)cyst detection by immunofluorescence assay (IFA), which was found to be essential to enumeration, as the live/dead test led to poor (oo)cyst labeling or a 10-fold lower recovery when carried out concomitantly to IFA. As for the viability assessment itself, although red dye uptake occurred as expected by dead or weakened organisms, neither live *G. muris* cysts or *C. parvum* oocysts present any green fluorescence by esterase metabolism. This may have been caused by low enzyme activity in the infective form and/or wall thickness of these parasites. The results do not exclude the possibility of simultaneous fluorescence staining for protozoa, but it is a starting point for a broader analysis, that may consider, for instance, different incubation conditions.

Keywords Viability assessment; pathogenic protozoa; propidium iodide; fluorescent dyes; cyst staining

Introduction

Cryptosporidium and *Giardia* are pathogenic protozoa responsible for many enteric diseases outbreaks in the world [1]. These pathogens prevail in the environment in their infective form, oocysts and cysts, respectively, which are able to survive for months in soil and surface water [2]. They also constitute resistant structures [3] mainly because *Cryptosporidium* spp. presents surface proteins [4] and *Giardia* spp. has a double-layered wall [5]. These organisms pose a challenge to public health because they are extremely resistant to chemical disinfection by chlorine, chloramines, and chlorine dioxide [6]. Even more invasive treatments, such as ozonation, do not yield complete inactivation of cysts [7] and ultraviolet (UV) systems provide less inactivation on these parasites compared to other indicators [8].

These characteristics make cysts and oocysts important target organisms when assessing disinfection efficiency, verified by their inactivation, which may not be a consistent parameter in defining if the organism is dead or alive [9]. The gold standard analysis to evaluate protozoa inactivation is animal infectivity; however, these tests are costly for routine examination and bring up ethical concerns towards research with animals. They also require a minimum concentration of cysts and oocysts, which might not be realistic depending on the environmental sample [10].

Viability assays, on the other hand, provide inferences based on molecular techniques [11–13], as well as imaging, particularly grounded on excystation [14,15], metabolic activity or cellular integrity determined by dye inclusion/exclusion [16–19]. Although labor-intensive and often impaired by artifacts in either flow cytometry or microscopy [20,21], viability methods are relevant in the environmental scenario. The main point is that intact and metabolically functional cysts and oocysts might not be necessarily infectious, as their sporozoites or trophozoites may fail to infect their hosts. This possibility may be disadvantageous from a parasitology perspective, reaffirming infectivity as the barometer in this topic [22]. Nevertheless, as viability may underestimate treatment efficiency, overestimating risk, it could be beneficial from a public health perspective [23].

Method 1623.1 [24] recommends using immunofluorescence assays (IFA) for detecting cysts and oocysts in water. Therefore, fluorescent dye-based viability assessments consist on a simple extra step in detection, which encourages its use, especially for laboratories

that lack infrastructure for molecular examination or infectivity tests. Some known fluorescent stains are ethidium and propidium dyes, which have high affinity for nucleic acids but are impermeable to cell membranes. Its incorporation occurs in a passive physical-chemical form, indicating if the cells are damaged and, consequently, staining nucleic material of organisms that may be non-viable, weakened or dead [9]. Propidium iodide (PI), for instance, has been used in viability assessment of protozoa detected in effluent from anaerobic reactors [18], sewage sludge [25] livestock residues [17] as well as water supply [16] and simulated water treatment residues [26].

Another fluorescent dye-based alternative for viability assessment are esterase substrates. In this scene, viability is inferred both by the enzymatic activity, which is required to generate fluorescence, and by the integrity of the cell membrane, which is necessary for the retention of fluorescent products within the cell. Because they are electrically neutral molecules, these esterase substrates freely penetrate cells by diffusion. Once inside the cells, these molecules are converted into fluorescent products by the intracellular enzyme, and are then retained by organisms with intact plasma membranes [9,27]. Carboxifluorescein diacetate (CFDA) is an example of this type of stain, which leads to green fluorescence in cells considered viable. This dye has been applied for assessing viability of bacteria in biofilms [9] as well as algae [28]. Similarly, SYTO-dyes uptake and their green fluorescence effects on considered viable *C. parvum* oocysts have correlated to infectivity results when evaluating the efficacy of UV, thermal treatments and oxidants [29–31].

In order to improve dye-based techniques and overcome possible deficiencies, the combination of vital dyes can be applied to associate both exclusion and inclusion methods in which a non-fluorescent dye is incorporated and metabolized to fluoresce in living cells, and another stain is incorporated by cells with damaged membranes [23]. This effect is expected by some commercial kits, which suggest the simultaneous visualization of cells considered viable and non-viable (i.e. within the same panel). This may therefore cover the entire sample and lead to cell enumeration coupled to viability estimation. These tests are often performed for bacteria [32,33] but there are reports on the application of live/dead assays on *Ascaris* spp. eggs [34,35], which represent a resistant indicator organism, encouraging this analysis on protozoa, for instance.

In this context, the aim of this study was to explore analytical challenges and perspectives of the application of a simultaneous vital dye analysis, using a live/dead commercial kit (ab115347, Abcam®, UK) as a method for assessing viability and enumerating purified samples of *Giardia muris* cysts and *Cryptosporidium parvum* oocysts. The performance of the live/dead assay, carried out in different conditions, was verified by comparing the simultaneous visualization panel (both green and red organisms present, live and dead, respectively) with the isolated dead cell filter (PI-filter, for red dead cells). Also, a parallel test was conducted as a control, in which propidium iodide (PI) was added to the suspensions and (oo)cyst were quantified by immunofluorescence.

Material and methods

Target organisms

The viability assays were performed over suspended *Giardia muris* cysts and *Cryptosporidium parvum* oocysts acquired live. Both suspensions (Waterborne^{TM}, Inc, USA) were extracted from experimentally infected Swiss Webster (CFW®) mice and calves, respectively, and purified from feces by sucrose and percoll density gradient centrifugation. The organisms were stored at 4 ºC in deionized water with penicillin, streptomycin, gentamicin, amphotericin B and 0.01% Tween® 20, following the supplier's recommendations. Viability tests and slide counts were carried out promptly, in order to prevent die-off of the organisms during the experiments. The (oo)cysts were not subjected to heat killing or any inactivation control in this study. There is often, however, a fraction of non-viable (oo)cysts in purified suspensions, even within early evaluation.

Sample preparation for viability assessment involved homogenizing each suspension by three cycles of two-minute vortexing followed by vigorous pipetting (using a disposable plastic Pasteur pipette). Aliquots of 5 µL of each suspension of cysts and oocysts were spiked together for the different methods tested, each of them performed in triplicate.

After slide preparation by different methods, the organisms were analyzed by incorporation of the different vital dyes. The organisms were enumerated according to dye uptake verified in different optical filters and a qualitative assessment was performed based on

procedure simplicity as well as organism visualization clarity. All materials exposed to protozoa were previously rinsed with Tween® 80 elution solution (0.1% v v^{-1}).

Simultaneous vital dye assay

The live/dead assay was put through using a commercial kit (ab115347, Abcam®, UK) according to the manufacturer's protocol for microscopy readings. Briefly, the concentrated reagent (1000X) was diluted to 5X (v v^{-1}) in phosphate buffer saline solution (PBS, pH 7.4). Solutions were prepared in 0.2 mL micro-centrifuge tubes (Eppendorf®) wrapped in aluminum foil for luminosity protection and promptly used after dilution.

Three different slide-mounting procedures were tested for the simultaneous vital dye assay, in order to exclude possible interferences in the stains performance. In all of the tested methods, the 5X concentrated reagent was overlaid to the suspensions in the same volumes of such. Slide preparation was carried out in the absence of direct light.

The (oo)cysts were visualized in an epifluorescence microscope (BX51, Olympus®) with a 40x objective. Live organisms were expected to present green fluorescence under maximum excitation of 495 nm and 520 nm emission, compatible with FITC. Organisms were considered dead (non-viable) by incorporating red dye, which, once bound to DNA, would increase red fluorescence under 617 nm and 528 nm maximum excitation and emission, respectively. This was expected to be observed under FITC and appear in bright red under the specific excitation filter (PI-filter).

Method A

The 5X concentrated live/dead reagent placed with the suspension aliquots was incubated for 10 min at room temperature. The volume consisted on 10 µL, corresponding to the inoculum (5 µL of *G. muris* and 5 µL of *C. parvum* suspensions). The reagent excess was removed by rinsing each well with 50 µl HBSS and 50 µL of ultrapure water.

An immunofluorescence assay (IFA) was carried out using the Merifluor® G/C kit (Meridian Bioscience Diagnostics, USA), according to the protocol established by the manufacturer. Some additional steps were included in order to perform the confirmatory test recommended by Method 1623.1 (USEPA, 2012). Therefore, after staining the

samples with the Merifluor® G/C reagents, two drops of Fluoroshield™ with DAPI (F6057, Sigma-Aldrich®) were added to each slide well and left for staining for 10 min. This was followed by three slide washings using 50 µl of Hank's Balanced Salt Solution (HBSS, H6648, Sigma-Aldrich®). A drop of Merifluor® mounting medium was added to each well and they were sealed with a slide coverslip.

Method B

A second live/dead cell assay was carried out in association with Fluoroshield™ with DAPI, excluding Merifluor® labels. This aimed to evaluate whether the Merifluor® fluorescence mechanisms would interfere the performance of green viability indicative reagents. The enumeration and viability assessment of (oo)cysts should be simultaneously possible under FITC (green = live, soft red = dead), while PI-filter should confirm "dead" organisms enhanced in intense red fluorescence against the background. DAPI-filter would work as a confirmatory test for morphology.

Method C

A third method was investigated by evaluating the live/dead reagent overlaid directly on the inoculum, without adding any other dyes. The samples were not washed with HBSS nor fixed with any mounting media. Both enumeration and viability assessment were expected to be carried out simultaneously, as indicated in the previous procedure.

Propidium iodide (PI) staining

PI (P4170, Sigma-Aldrich®) solution (1.0 mg L^{-1}) was prepared in the absence of direct light and stored at 4 ºC in an amber bottle wrapped in aluminum foil. PI was added to the inoculum (same volume as the sample, i. e. 10 µL to overlay both suspensions) and was left for staining for 5 min in absence of direct light. The slide was then rinsed with 50 µl of HBSS and 50 µL of ultrapure water, in order to avoid the formation of crystals, as well as to remove the excess of dye. Microscopy slides were prepared for immunofluorescence assay (IFA) by applying the Merifluor® G/C kit protocol, including Fluoroshield™ with DAPI staining, accordingly to Method 1623.1 (USEPA, 2012).

Enumeration was carried out under FITC-fluorescence, considering organisms labeled with Merifluor® G/C. The viability estimation based on observing target organisms that would incorporate PI and should be visible under excitation around 500 nm, compatible to the optical filter applied for "dead" cells on the previous assay. Red-stained cysts and oocysts were considered non-viable, whilst those that did not incorporate the stain but were detected by their characteristic FITC-fluorescence (bright apple green) when treated for IFA were taken as viable organisms.

Data analysis

Regarding simultaneous staining, cysts and oocysts were expected to be visualized and have their viability assessed within the same panel, under FITC-filter, for all methods. In method A, FITC-filter range of excitation and emission should allow the visualization of non-viable (oo)cysts with their walls stained with green, by reacting with Merifluor® G/C reagents, and some red or orange color within the interior of the cysts. Viable organisms were expected to be entirely green, for wall labeling and esterase activity. When analyzing the same treatment under PI-filter, only red (non-viable) parasites should be visible. Results from methods B and C should lead to the same effect, but considering that green (oo)cysts under FITC-filter would be viable, as their fluorescence should be only and directly related to esterase activity.

(Oo)cyst quantification was analyzed in order to verify if all of the organisms present under FITC-filter (viable and non-viable) were compatible to the sum of green (viable) cysts under FITC-filter and saturated red under PI-filter (non-viable). This comparison should provide a notion of heterogeneous dye uptake, as well as indicate if viability assessment could be performed within the same visualization filter as enumeration, which would lead to a more rapid and straightforward analysis. These results were submitted to the Shapiro-Wilk normality test ($\alpha = 0.05$) and transformations were performed, if necessary, so the data could be tested for the null hypothesis of similar mean visualization. The software PAST [36] was used to apply a paired Student t-test (95% confidence interval).

Enumeration data, as well as viability estimation obtained for the simultaneous staining was also analyzed comparatively to the one obtained for the immunofluorescence assay (IFA) coupled to propidium iodide (PI) staining.

Results and discussion

Performance of the different methods of simultaneous live/dead staining

Results obtained from method A (Table 1) were consistent for *C. parvum*, as no numerical evidence of difference in visualization of simultaneous and compared filters were noticed. This suggests oocysts could be enumerated and have their viability assessed in one panel. However, the obtained data led to ambiguities caused by heterogeneous dye uptake for *G. muris*. The sum of *G. muris* cysts identified under FITC-fluorescence in both green and soft red did not correspond to the total of organisms observed in green under FITC and bright red under the PI-filter. Descriptive statistics indicated variability within repetitions, which is noteworthy as the experiments were carried out with purified suspensions, whilst environmental samples contain debris that impair (oo)cyst recovery and visualization [37] and may therefore affect viability estimation even more substantially. Although more replications could improve inferences in the methodological aspect of this research, costs for immunofluorescence detection are restrictive [38].

When extrapolating results by univariate statistics, however, both single-panel and two-panel microscopy quantifications for *G. muris* were normally distributed (Shapiro-Wilk test, *p*-value = 0.576 and *p*-value = 0.486, respectively) and no outliers were found. Similarly, the numbers of oocysts were normally distributed (*p*-value = 0.789). Student's t-test for equal means did not lead to significant differences for visualization in FITC and PI filters for *G. muris* cysts (*p*-value = 0.529) nor *C. parvum* oocysts (*p*-value = 1.00). These preliminary results suggest potential for the simultaneous visualization methodology.

Table 1 – Enumeration of cysts and oocysts identified by method A for viability assessment, using Merifluor®, Fluoroshield™ with DAPI and the Live/Dead Cell Assay (Abcam®)

Microscopy	**Dye uptake**	***G. muris* cysts**	**Mean ±**	***C. parvum***	**Mean ±**

filter					SD	oocysts			SD
		n1	n2	n3		n1	n2	n3	
FITC	Green (live)	23	41	17	27±10	72	82	22	59±26
	Red (dead)	78	92	70	80±9	2	3	2	2±0,5
PI	Red (dead)	90	123	78	97±19	2	3	2	2±0,5
FITC (one-panel visualization)	Green (live) + red (dead)	101	133	87	107±19	74	85	24	61±27
FITC + PI (two-panel visualization)	Green (live in FITC-filter) + red (dead in PI-filter)	113	164	95	124±29	74	85	24	61±27

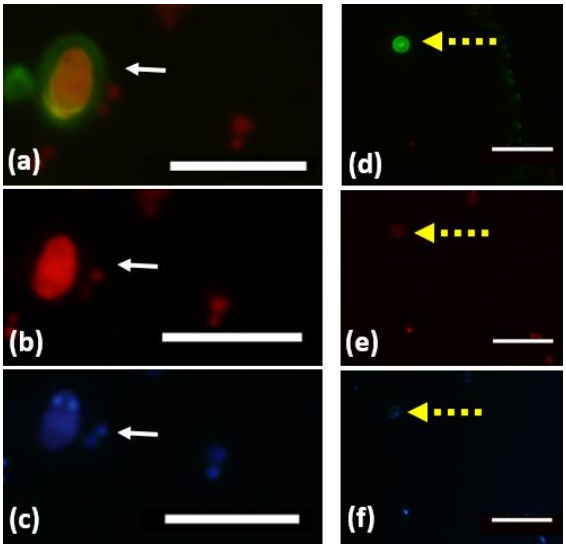
Notes: Values indicate number of detected (oo)cysts. SD = standard deviation. Each "n" represents a repetition. Sample volume: 5µL.

As of imaging, Fig. 1 (a, b c) displays an example of a considered non-viable *G. muris* cyst, which presented a green rim under FITC-filter, expected to derive from the immunofluorescence assay (IFA) effect, and red fluorescence under PI-filter. A viable oocyst is also present in Fig 1 (d, e, f), pointed by the dashed arrows, and the entire organism is green under FITC-fluorescence, which probably relates from both wall labeling from IFA and esterase activity. Under PI-filter (Fig 1, e), the viable *C. parvum* oocyst is not visible, an indicator of membrane integrity.

It is worth mentioning that most *G. muris* cysts did not exhibit bright apple green fluorescence under FITC-filter as expected by following Method 1623.1 [24] with Merifluor® G/C reagents for cysts and oocysts detection. Research developed by Alderisio et al. [39], in which commercially available staining kits to detect *Giardia* spp. were tested for their use with Method 1623.1 [24] showed very poor fluorescence signals on *G. muris* either stained with EasyStain™ (BTF Precise Microbiology, USA), Aqua-Glo™ (Waterborne Inc., USA) or Merifluor® (Meridian Bioscience Diagnostics, USA). Although it is possible to acquire specific antibodies with fluorophores for *G. muris*, there is no point in isolated analysis from an environmental monitoring or sanitation perspective, where these commercial kits are often employed. This poor fluorescence affects in more or less conservative estimation of occurrences and densities of zoonotic *Giardia* cysts, as it is dependent on species/assemblages [39]. Thus, considering the viability assessment from the live/dead simultaneous kit is not specific, any enhancement in green under FITC-fluorescence would be favorable to detecting enzyme active *G. muris* cysts that may not

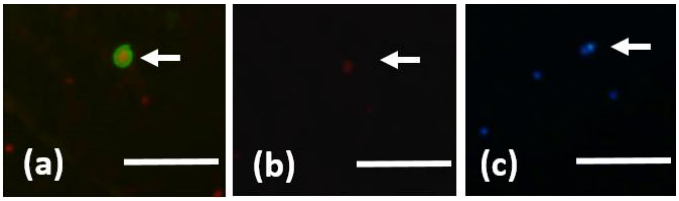
have had their walls adequately labelled by IFA, supporting enumeration alongside to viability estimation.

Fig 1 – *Giardia muris* cyst considered non-viable after staining by Method A imaged under (a) FITC-filter; (b) PI-filter, and; (c) DAPI-filter and, *Cryptosporidium parvum* viable oocyst under (d) FITC-filter; (e) PI-filter, and; (f) DAPI-filter. Scale bar = 20μm.



The single-panel assessment, however, may be subjective. It is important to note it relies much on the microscopist, as heterogeneous dye incorporation may lead to doubts, particularly on what concerns oocysts, that are small in size (approximately 5 μm width [40]). This might hinder distinguishing whether green fluorescence is occurring within the organism or only on its wall or if reddish dye uptake refers to debris. Fig. 2 displays an example of this situation happening in method A, where a *C. parvum* oocyst seems to have its interior stained in red (a wall damage indicator) and its wall marked by IFA. A single-panel simultaneous analysis should lead to an interpretation of non-viability, but the organism does not present any red fluorescence under PI-filter, where only "dead" cells should appear.

Fig 2 – *C. parvum* oocyst after staining by Method A (a) seemingly non-viable under FITC-filter; (b) not present under PI-filter, and; (c) not distinguishable under DAPI-filter. Scale bar = 20μm.

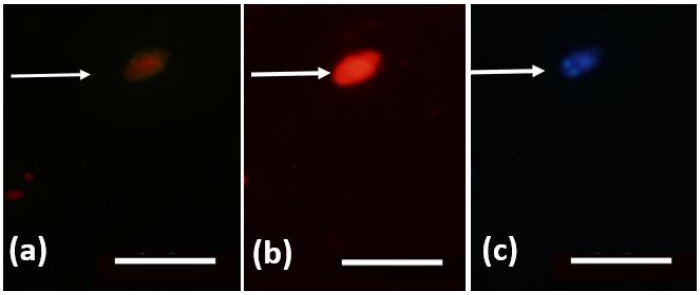


Groups of organisms within a microbial population may stain heterogeneously and thus be arranged into more subcategories between live and dead. [41]. An example of this subjective interpretation was verified by Davey and Heley [42], who identified that different stress levels lead to PI-uptake in yeasts, although that did not necessarily mean that cells were non-viable, because they were able to repair membrane damage after incubation. Karkashan et al. [35], when assessing viability of *Ascaris* spp. eggs with BacLight® reported similar inaccuracies in viability determination, which were assumed to be caused by variable degradation levels of proteins in the lipoprotein layer of the organisms, leading to uncertain classifications.

As for protozoa, some studies [43,44] reported related discrepancies in viability assessment between dye penetration methods and infectivity assays for *C. parvum* oocysts. *G. muris* cysts, as a model for *G. duodenalis*, presented a similar effect when Schupp and Erlandsen [45] compared fluorescein diacetate (FDA) in conjunction with PI with animal infectivity tests. Smith and Smith [46] also verified *G duodenalis* cysts that stained in red by PI could also present green fluorescence by FDA. This could be explained by enzymes that remain active even in organisms that do not exhibit complete membrane integrity [23].

Neither methods B or C, in which Merifluor® reagents were not included, allowed visualizing viable organisms, expected to present green fluorescence under FITC-filter due to esterase activity. Under DAPI-filter (Method B), some *G. muris* cysts were detected because of their stained nuclei and they were either considered non-viable (if presenting red stain uptake under PI-filter) or they were not visualized showing green fluorescence. An example of this effect is indicated by an arrows in Fig. 3, which displays a considered non-viable *G. muris* cysts, that did not present any green fluorescence under FITC (Fig 3, panel a), but stained brightly in red under PI-filter (Fig 3, b). Nevertheless, (oo)cyst identification and detection should not rely on DAPI filter, as Method 1623.1 [24] recommends it only for morphology confirmation after immunofluorescence specific visualization, which is antibody-based.

Fig 3 – *G. muris* cysts considered non-viable after staining by Method B. Visualization under (a) FITC-filter; (b) PI-filter, and; (c) DAPI-filter. Scale bar = 20μm.



C. parvum oocysts were not detected in the samples, as DAPI-filter is only a confirmatory test and it is very sensitive to debris interference in the microscopy well, and oocysts did not present any green fluorescence under FITC-filter. Red stain uptake occurred as expected for cysts and oocysts considered non-viable, which were identified under PI-filter. Therefore, the simultaneous vital dye assessment was not efficient for replacing IFA in cysts and oocysts enumeration in a single-panel analysis, due to the lack of green fluorescence in supposedly viable organisms.

It was inferred that there was low uptake of the esterase substrate that should lead to green staining of viable (oo)cysts. Accordingly, Santos et al. [48] stated that even fresh and intact samples of *G. duodenalis* and *C. parvum*, because of their robust cell walls [40], may not be labeled by fluorescent molecules. Examples of such are CFDA-SE (carboxyfluorescein succinimidyl ester diacetate), similar to carboxyfluorescein diacetate (CFDA), both common viability assessment dyes. Although they are fluorescein-based, detection reagents like Merifluor® have surface action when marking cysts and oocysts, i. e. there is an immunofluorescence reaction and the organisms are marked on their walls. As for a live/dead viability assay, supposedly living cells need to incorporate the non-fluorescent dye by diffusion and then metabolize it in order to present fluorescence, coming from an product retained inside of the viable cell.

Comparisons to propidium iodide control experiment

Table 2 presents the results for cysts and oocysts enumeration after slide preparation by Method 1623.1 [24] adding propidium iodide (PI). This methodology, which was similar to Method A using the simultaneous staining, lead to PI uptake in line with what was expected, i. e. parasites were detected under FITC excitation and emission ranges and those which presented red staining under PI-filter were taken as non-viable. In addition, the method was operationally simple to carry out. However, the total enumerated organisms by Merifluor® labeling (green-marked cysts and oocysts within the FITC panel), was higher

(approximately 10^1 times) than what was verified for slide preparation using the Live/Dead Cell Assay (Abcam®) in method A, when counting the same suspension volumes, for both species under analysis (table 1). This endorses a possible influence of simultaneous staining on the Merifluor® performance, which may compromise the counting of cysts and oocysts.

Table 2 – Enumeration of *G. muris* and *C. parvum* (oo)cysts by Method 1623.1 (USEPA, 2012) in addition to propidium iodide (PI) staining for viability assessment

Filter	**Fluorescence**	***G. muris***			**Mean ± SD**	***C. parvum***			**Mean ± SD**
		n1	**n2**	**n3**		**n1**	**n2**	**n3**	
FITC	Green	420	959	945	745±251	262	467	350	360±84
PI	Red (non-viable)	418	1079	947	815±286	85	130	57	91±30

Notes: Organisms visualized in red under PI-filter are considered non-viable. SD = standard deviation. Each "n" represents a repetition. Sample volume: 5µL.

The results found in this study do not exclude the possibility of simultaneous staining methods for viability assessment work on protozoa, especially because there was red-stain uptake. Optimizing detection and viability protocols, which lack objective standards [49], consist on a fundamental task, as steps might differ for each organism, encouraging research on different strains and that could also more replications, when feasible. For instance, in order to develop a less labor-intensive method for assessing viability of helminth eggs in sewage sludge, a live/dead bacterial kit (BacLight®, Molecular Probes Inc., USA) was used by Dabrowska et al. [34]. The study indicated that the kit allowed distinguishing viable from non-viable eggs, as the authors tested different variants of dyes mixture in concentration. The method was also tested by Karkashan et al. [35], comparing it to conventional incubation and other vital stains, providing a satisfactory performance. The fundamental principle of BacLight® kit is the same as Live/Dead Cell Assay's (Abcam®) used in the present study. The fact that it was efficient on resistant organisms such as helminth eggs, often used as indicators [50], encourage further investigation on simultaneous dye-based viability assays on protozoa.

Conclusions

Comparatively to (oo)cyst enumeration and viability assessment from a standard immunofluorescence assay (IFA) protocol coupled to propidium iodide (PI) inclusion test, this study demonstrated that IFA reagents have their efficiency impaired by the Live/Dead Cell Assay (Abcam®) kit.

Appling the simultaneous stains without IFA reagents, however, is not recommended for (oo)cyst enumeration, considering supposedly viable *G. muris* cysts and *C. parvum* oocysts did not lead to any green fluorescence from esterase activity in the simultaneous live/dead test. The organisms considered non-viable incorporated red dye, presenting a similar effect of propidium iodide conventional staining. The single-panel analysis for viability assessment and enumeration was therefore not efficient in this study.

Nevertheless, further investigation of simultaneous staining for live/dead assessment in protozoa is encouraged. Testing different incubation conditions and dye concentrations are particularly recommended, in order to stimulate esterase substrates incorporation as well as enzymatic activity.

Disclosure statement

The authors reported no potential conflict of interest.

Funding

The São Paulo Research Foundation (FAPESP) under Grant 12/50522-0 and the Global Challenges Research Fund (GCRF) UK Research and Innovation (SAFEWATER; EPSRC Grant Reference EP/P032427/1) supported this work. The Coordination for the Improvement of Higher Education Personnel (CAPES-PROEX – Financial code 001) granted Kamila Jessie Sammarro Silva with a Master's scholarship.

References

[1] Efstratiou A, Ongerth J, Karanis P. Evolution of monitoring for *Giardia* and *Cryptosporidium* in water. Water Res. 2017;123:96–112.: http://dx.doi.org/10.1016/j.watres.2017.06.042.

[2] Gallas-Lindemann C, Sotiriadou I, Plutzer J, et al. Prevalence and distribution of *Cryptosporidium* and *Giardia* in wastewater and the surface, drinking and ground waters in the Lower Rhine, Germany. Epidemiol. Infect. 2013;141:9–21.

[3] Bouzid M, Hunter PR, Chalmers RM, et al. *Cryptosporidium* pathogenicity and virulence. Clin. Microbiol. Rev. 2013;26:115–134.

[4] Templeton TJ. Comparative Analysis of Apicomplexa and Genomic Diversity in Eukaryotes. Genome Res. 2004;14:1686–1695. http://www.genome.org/cgi/doi/10.1101/gr.2615304.

[5] Erlandsen SL, Macechko PT, Keulen HV, et al. Formation of the *Giardia* Cyst Wall: Studies on Extracellular Assembly Using Immunogold Labeling and High Resolution Field Emission SEM. J. Eukaryot. Microbiol. 1996;43:416–430. http://doi.wiley.com/10.1111/j.1550-7408.1996.tb05053.x.

[6] Korich DG, Mead JR, Madore MS, et al. Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability. Appl. Environ. Microbiol. 1990;56:1423–1428.

[7] Kondo Nakada LY, Bueno Franco RM, da Silva Fiuza VR, et al. Pre-ozonation of source water: assessment of efficacy against *Giardia duodenalis* cysts and effects on natural organic matter. Chemosphere 2018;214:764–770. https://www.sciencedirect.com/science/article/pii/S0045653518318307.

[8] Li H, Jin Y, Nie Y. Bioresource Technology Application of alkaline treatment for sludge decrement and humic acid recovery. Bioresour. Technol. 2009;100:6278–6283. Available from: http://dx.doi.org/10.1016/j.biortech.2009.07.022.

[9] Netuschil L, Auschill TM, Sculean A, et al. Confusion over live/dead stainings for the detection of vital microorganisms in oral biofilms - which stain is suitable? BMC Oral Health. 2014;14:1–12.

[10] Koehler A V., Jex AR, Haydon SR, et al. *Giardia*/*Giardia*sis - A perspective on diagnostic and analytical tools. Biotechnol. Adv. [Internet]. 2014;32:280–289. Available from: http://dx.doi.org/10.1016/j.biotechadv.2013.10.009.

[11] Lee G-C, Nam S-H, Chae J-C, et al. *Giardia* duodenalis: Improved detection of viable cysts by reverse transcription-PCR of heat shock-inducible hsp70 gene. Exp. Parasitol. [Internet]. 2009;123:377–380. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0014489409002379.

[12] Brescia CC, Griffin SM, Ware MW, et al. Cryptosporidium propidium monoazide-PCR, a molecular biology-based technique for genotyping of viable Cryptosporidium oocysts. Appl. Environ. Microbiol. 2009;75:6856–6863.

[13] Paziewska-Harris A, Schoone G, Schallig HDFH. An easy ‘one tube’ method to estimate viability of *Cryptosporidium* oocysts using real-time qPCR. Parasitol. Res. 2016;115:2873–2877. http://dx.doi.org/10.1007/s00436-016-5044-z.

[14] Pecková R, Stuart PD, Sak B, et al. Statistical comparison of excystation methods in *Cryptosporidium parvum* oocysts. Vet. Parasitol. 2016;230:1–5.

[15] Smith H V., Nichols RAB, Grimason AM. *Cryptosporidium* excystation and invasion: Getting to the guts of the matter. Trends Parasitol. 2005;21:133–142.

[16] Gómez-Couso H, Fontán-Sainz M, McGuigan KG, et al. Effect of the radiation intensity, water turbidity and exposure time on the survival of *Cryptosporidium* during simulated solar disinfection of drinking water. Acta Trop. 2009;112:43–48.

[17] Grit GH, Bénéré E, Ehsan A, et al. *Giardia duodenalis* cyst survival in cattle slurry.

Vet. Parasitol. 2012;184:330–334. http://dx.doi.org/10.1016/j.vetpar.2011.08.021.

[18] Kinyua MN, Trimmer J, Izurieta R, et al. Viability and fate *of Cryptosporidium parvum* and *Giardia lamblia* in tubular anaerobic digesters. Sci. Total Environ. 2016;554–555:167–177. http://dx.doi.org/10.1016/j.scitotenv.2016.02.170.

[19] Robertson LJ, Casaert S, Valdez-Nava Y, et al. Drying of *Cryptosporidium* oocysts and *Giardia* cysts to slides abrogates use of vital dyes for viability staining. J. Microbiol. Methods. 2014;96:68–69. http://dx.doi.org/10.1016/j.mimet.2013.11.001.

[20] Vesey G, Hutton P, Champion A, et al. Application of flow cytometric methods for the routine detection of Cryptosporidium and *Giardia* in water. Cytometry 1994;16:1–6. http://doi.wiley.com/10.1002/cyto.990160102.

[21] Nie X, Liu W, Chen M, et al. Flow cytometric assessment of the effects of chlorine, chloramine, and UV on bacteria by using nucleic acid stains and 5-cyano-2,3-ditolyltetrazolium chloride. Front. Environ. Sci. Eng. 2016;10:12. http://link.springer.com/10.1007/s11783-016-0884-4.

[22] Bukhari Z, Marshall MM, Korich DG, et al. Comparison of *Cryptosporidium parvum* viability and infectivity assays following ozone treatment of oocysts. Appl. Environ. Microbiol. 2000;66:2972–2980.

[23] Rousseau A, La Carbona S, Dumètre A, et al. Assessing viability and infectivity of foodborne and waterborne stages (cysts/oocysts) of *Giardia duodenalis*, *Cryptosporidium* spp., and *Toxoplasma gondii*: a review of methods. Parasite 2018;25:14. https://www.parasite-journal.org/10.1051/parasite/2018009.

[24] USEPA. Method 1623.1: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA. United States Prot. Agency. US Environmental Protection Agency, Office of Water; 2012. p. 83. http://water.epa.gov/scitech/drinkingwater/labcert/upload/epa816r12001.pdf.

[25] Medeiros RC, Daniel LA. Quantification and analysis of the viability of (oo)cysts of pathogenic protozoa in sewage sludge. Acta Sci. Technol. 2018;40:28709. http://periodicos.uem.br/ojs/index.php/ActaSciTechnol/article/view/28709.

[26] Sammarro Silva KJ, Sabogal-Paz LP. *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts in drinking water treatment residues: comparison of recovery methods for quantity assessment. Environ. Technol. 2020;0:1–10. https://doi.org/10.1080/09593330.2020.1723712.

[27] Iturriaga R, Zhang S, Sonek GJ, et al. Detection of respiratory enzyme activity in *Giardia* cysts and *Cryptosporidium* oocysts using redox dyes and immunofluoresce techniques. J. Microbiol. Methods. 2001;46:19–28.

[28] MacIntyre HL, Cullen JJ. Classification of phytoplankton cells as live or dead using the vital stains fluorescein diacetate and 5-chloromethylfluorescein diacetate. J. Phycol. 2016;52:572–589.

[29] Finch GR, Black EK, Labatiuk CW, et al. Comparison of *Giardia lamblia* and *Giardia muris* cyst inactivation by ozone. Appl. Environ. Microbiol. 1993;59:3674–3680.

[30] Belosevic M, Guy TTRA, Gyorizk LL, et al. Nucleic acid stains as indicators of *Cryptosporidium parvum oocyst* viability. Int. J. Parasitol. 1997;27:787–798.

[31] Neumann NF, Gyürek LL, Gammie L, et al. Comparison of animal infectivity and nucleic acid staining for assessment of *Cryptosporidium parvum* viability in water. Appl. Environ. Microbiol. 2000;66:406–412.

[32] Hu W, Murata K, Zhang D. Applicability of LIVE/DEAD BacLight stain with glutaraldehyde fixation for the measurement of bacterial abundance and viability in rainwater. J. Environ. Sci. (China). 2017;51:202–213. http://dx.doi.org/10.1016/j.jes.2016.05.030.

[33] Boulos L, Prévost M, Barbeau B, et al. LIVE/DEAD® BacLight: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J. Microbiol. Methods. 1999;37:103–104.

[34] Dabrowska J, Zdybel K, Karamon J, et al. Assessment of viability of the nematode eggs (Ascaris, Toxocara, Trichuris) in sewage sludge with the use of Live/Dead Bacterial Viability Kit. Agric. Environ. Med. 2014. p. 35–41.

[35] Karkashan A, Khallaf B, Morris J, et al. Comparison of methodologies for enumerating and detecting the viability of Ascaris eggs in sewage sludge by standard incubation-microscopy , the BacLight Live/Dead viability assay and other vital dyes. Water Res. 2014;68:533–544. http://dx.doi.org/10.1016/j.watres.2014.10.003.

[36] Hammer Ø, Harper DA, Ryan PD. PAST: paleontological statistics software package for education and data analysis. Palaeontol. Electron. 2001;4.

[37] Ongerth JE. The concentration of *Cryptosporidium* and *Giardia* in water – The role and importance of recovery efficiency. Water Res. 2013;47:2479–2488. http://dx.doi.org/10.1016/j.watres.2013.02.015.

[38] Andreoli FC, Sabogal-Paz LP. Coagulation, flocculation, dissolved air flotation and filtration in the removal of *Giardia* spp. and *Cryptosporidium* spp. from water supply. Environ. Technol. 2019;40:654–663. https://www.tandfonline.com/doi/full/10.1080/09593330.2017.1400113.

[39] Alderisio KA, Villegas LF, Ware MW, et al. Differences in staining intensities affect reported occurrences and concentrations of *Giardia* spp. in surface drinking water sources. J. Appl. Microbiol. 2017;123:1607–1613.

[40] Carey CM, Lee H, Trevors JT. Biology, persistence and detection of Cryptosporidium parvum and *Cryptosporidium hominis* oocyst. Water Res. 2004;38:818–862.

[41] Davey HM. Life, Death, and In-Between: Meanings and Methods in Microbiology. Appl. Environ. Microbiol. 2011;77:5571–5576. http://aem.asm.org/lookup/doi/10.1128/AEM.00744-11.

[42] Davey HM, Hexley P. Red but not dead? Membranes of stressed *Saccharomyces cerevisiae* are permeable to propidium iodide. Environ. Microbiol. 2011;13:163–171. http://doi.wiley.com/10.1111/j.1462-2920.2010.02317.x.

[43] Lorenzo-Lorenzo MJ, Ares-Mazas ME, de Maturana IV-M, et al. Effect of Ultraviolet Disinfection of Drinking Water on the Viability of *Cryptosporidium parvum* Oocysts. J. Parasitol. 1993;79:67. https://www.jstor.org/stable/3283279?origin=crossref.

[44] Kato S, Jenkins M, Fogarty E, et al. Effects of freeze-thaw events on the viability of *Cryptosporidium parvum* oocysts in soil. J. Parasitol. 2002;88:718–722. http://www.scopus.com/inward/record.url?eid=2-s2.0-0036340970&partnerID=40%5Cnpapers2://publication/uuid/A6AD7E86-4B0D-4536-8398-1EEC99186D69.

[45] Schupp DG, Erlandsen SL. A new method to determine *Giardia* cyst viability: Correlation of fluorescein diacetate and propidium iodide staining with animal infectivity. Appl. Environ. Microbiol. 1987;53:704–707.

[46] Smith AL, Smith H V. A comparison of fluorescein diacetate and propidium iodide staining and in vitro excystation for determining *Giardia intestinalis* cyst viability. Parasitology. 1989;99:329–331.

[47] Santos SR Dos, Branco N, Franco RMB, et al. Fluorescence decay of dyed protozoa: Differences between stressed and non-stressed cysts. Luminescence. 2015;30:1139–1147.

[48] Santos LU, Bonatti TR, Cantusio Neto R, et al. Occurrence of *Giardia* cysts and *Cryptosporidium* oocysts in activated sludge samples in Campinas, SP, Brazil. Rev. Inst. Med. Trop. Sao Paulo. 2004;46:309–313. http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0036-46652004000600003&lng=en&tlng=en.

[49] WHO. Integrated guide to: Sanitary Parasitology. Amman, Jordan: World Health Organization, Regional Office for the Eastern Mediterranean; 2004. p. 120.

[50] Bonatti TR, Franco RMB. Real scale environmental monitoring of zoonotic protozoa and helminth eggs in biosolid samples in Brazil. J. Parasit. Dis. 2016;40:633–642.