Water Research 189 (2021) 116590

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Review

Cryptosporidium spp. and *Giardia* spp. (oo)cysts as target-organisms in sanitation and environmental monitoring: A review in microscopy-based viability assays

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

ARTICLE INFO

Article history:
Received 29 June 2020
Revised 22 October 2020
Accepted 31 October 2020
Available online 2 November 2020

Keywords:
Vital dyes
Excystation
Fluorescence reduction
Pathogenic protozoa

ABSTRACT

Cysts and (oo)cysts are the infective forms of parasitic protozoa, as *Giardia* and *Cryptosporidium*, which are widespread and associated to worldwide waterborne diseases outbreaks. These microorganisms pose a challenge to public health, as they are resistant to conventional disinfection methods, which make them important parameters when evaluating inactivation efficiency. However, when (oo)cysts are targets, it is challenging to infer inactivation efficacy, as it may require infectivity tests that are not often an option for laboratory routine analysis. In this scene, (oo)cyst viability based on induced excystation, membrane integrity and enzyme activity evaluated by dye inclusion and/or exclusion, as well as fluorescence reduction consist on microscopy-based techniques that may be options to estimate inactivation in the environmental context. This scoping review presents applications, advantages and limitations of these methodologies for viability assessment, in order to shed light on the (oo)cyst viability topic and provide insight strategies for choosing protocols in the environmental and sanitation field, in laboratory applications and novel research.

1. Introduction and background

The importance of protozoan cysts and (oo)cysts as targets in environmental research and sanitation lies on the fact that *Giardia* spp. and *Cryptosporidium* spp. are pathogenic microorganisms associated to worldwide gastrointestinal diseases outbreaks (Efstratiou et al., 2017b), causing nutritional imbalances and mortality, particularly in children and immunologically deficient people (Thompson and Ash, 2016). There are several reports of these parasites in residues (Graczyk et al., 2008) and wastewater (Fernandes et al., 2011; Ramo et al., 2017a). Given the waterborne disease character of giardiasis and cryptosporidiosis, their aetiological agents have also been detected in both surface (Castro-Hermida et al., 2009; Chuah et al., 2016; de Araújo et al., 2018) and groundwater (Chique et al., 2020). A major concern regards occurrence in water treatment plants and general water sources (Kumar et al., 2016; Onichandran et al., 2014; Ramo et al., 2017b). A recent study highlighted the incidence of *Giardia* spp. and *Cryptosporidium* spp. in water for reuse, despite undertaking treatment (Razzolini et al., 2020). Drinking water sources in rural areas have also been recently reported with contamination with these parasites, which points this topic as a current matter (Kifleyohannes and Robertson, 2020).

The *Cryptosporidium* life cycle is completed in a single host and includes stages of sexual and asexual reproduction, detailed by Cacciò and Widmer (2014). The infection is acquired through the ingestion of oocysts. In the small intestine, motile sporozoites, from the open oocyst, attach to intestinal epithelial cells and become trophozoites. These undergo asexual replication, producing merozoites), which are released into the intestinal lumen and infect new cells. The merozoites may also go through a sexual cycle, which will result in a zygote that undergoes sporogony. The life cycle is complete when infectious sporulated oocysts are excreted in the faeces.

As for *Giardia*, the contamination also occurs by oral ingestion of cysts, which will break and release trophozoites in the upper parts of the small intestine, where they adhere to. The trophozoites multiply by binary division in the lumen. Finally, exposure to bile salts leads to encystation and the cysts are eliminated in faeces, closing the life cycle (Cacciò and Sprong, 2011; Geurden and Olson, 2011).

Infected individuals provide a large input of (oo)cysts into the environment and these are strongly persistent, as they may

* Corresponding author.
E-mail address: lysaboga@sc.usp.br (L.P. Sabogal-Paz).

https://doi.org/10.1016/j.watres.2020.116590
0043-1354/

remain infectious for extended times in different environmental conditions (Cama and Mathison, 2015; Jenkins et al., 1997; Yaoyu and Xiao, 2011). They are also resistant to various treatments (Kniel et al., 2004; Robertson et al., 1992; Ryan et al., 2016). These characteristics, added to the reported low infection dose (Boyer et al., 2009; Carey et al., 2004; DuPont et al., 1995), are thereby a major health concern, which qualifies infectivity and viability assessments as secondary objectives to (oo)cyst monitoring in environmental samples (Efstratiou et al., 2017a).

The ability of the parasite to complete its life cycle in the host, leading to disease, is called infectivity. It is determined by cell culture infection or bioassays, which are often not suitable for laboratory routine analyses in sanitation and environmental monitoring with frequent sampling. Animal testing, from an operational point of view, is considered costly, raises ethical issues and lacks reliability when low replications are used (Koehler et al., 2014; Rousseau et al., 2018).

Viability, in turn, is an inference about the amount of living cells based on their cellular integrity or metabolic activity. However, there is some imprecision in defining viable states of organisms, which leads to debates at different levels, as cells may present stages between "live" and "dead" (Davey, 2011; Netuschil et al., 2014). For instance, metabolically functional and intact protozoan (oo)cysts may fail to turn into sporozoites/trophozoites or infect their hosts (Rousseau et al., 2018). Hence, this type of examination may underestimate treatment efficiency (Buhkari et al., 2000) and overestimate risk. This brings up a controversial topic, because as much as some may claim it is “advantageous” from an environmental and public health perspective, by taking conservative approach; it could be counter-productive in establishing treatment efficacy. Therefore, viability estimation should ideally correlate to bioassays, so that detection of viable organisms that are not infective is minimized, and health risk assessment is reliable and straightforward. Despite no standard methodology is currently available, there is a wide range of methods either DNA/RNA- or microscopy-based, whose reliability will depend on how appropriate they are to the research in question.

Viability may be inferred by molecular techniques (Brescia et al., 2009; Lee et al., 2009; Paziewska-Harris et al., 2016), as well as imaging, often performed by microscopy visualization. Although the latter may suggest direct reliability on the microscopist, defining a protocol and established categories for inactivation stages of (oo)cysts would lead to strategic insight in automated detection coupled with viability assessment, which makes microscopy-based methods still stand out. Recently, there have been headways in pattern recognition in many areas, including rapid detection and quantification of *Giardia* spp. and *Cryptosporidium* spp. in water, assessed by artificial intelligence algorithms (Badsha et al., 2013; Koydemir et al., 2015). Nonetheless, there is a knowledge gap reported for protozoa viability data in environmental samples (Chique et al., 2020), which could be narrowed by adding image-based viability features to, for instance, machine-learning applications, a trend in predicting contamination (Ligda et al., 2020), but that could also be applied for assessing it. This could provide innovative technology for environmental monitoring and assessment, as in simple automated devices for routine analyses.

As for laboratory methodologies that lead to imaging, cysts and oocysts viability may be inferred by in vitro excystation (Pecková et al., 2016; Smith et al., 2005) and staining. Stain-based methods are: dye inclusion/exclusion (Gómez-Couso et al., 2009; Grit et al., 2012; Kinyua et al., 2016; Robertson et al., 2014) and fluorescence reduction after labelling (Guimarães et al., 2014; Kondo Nakada et al., 2018; Santos et al., 2015). This scoping review aimed to present situations in which these methodologies have been applied. Additionally, it covered advantages and limitations of different microscopy-based methods for viability assessment of protozoan (oo)cysts, when they are addressed as targets for environmental monitoring, as well as in the sanitation field.

2. Excystation methods

Pathogenic protozoa are found in the environment in their infective form, (oo)cysts, targeted for removal and/or inactivation, from the sanitation perspective. When ingested, their life cycles lead to sporozoites and trophozoites, after exposed to body temperature, acid, trypsin and bile salts (Smith et al., 2005). The former, which are banana-shaped organisms released from oocysts, refer to a life stage of *Cryptosporidium* spp., while the latter refers to *Giardia* spp. These forms are expected to be excystated, i. e. released from (oo)cysts, within the hosts if the infective cysts and oocysts are viable for infection. However, there is a lack in reproducible culture methodologies capable of supporting the parasite life cycle long term, pointing excystation as a key-process to prepare these pathogens for infection (Bones et al., 2019).

Considering that excystation is necessary for giardiasis and cryptosporidiosis to take place in an individual (Einarsson et al., 2016), performing this process in vitro may provide some understanding in pathogen viability, as well a step towards significant progress in the control of these diarrhoeal diseases (Boucher and Gillin, 1990; Karanis, 2018). In vitro assays mimic the environmental conditions in which excystation would occur. This process is carried out by exposing cysts and oocysts to an acid medium and subsequent incubating the microorganisms in bile salts, reducing agents and protease enzymes at 37 ºC. Excystation methods therefore imitate the conditions to which the parasites would be subjected in the transition between the stomach and the intestines of the host, which is why they may be performed concomitantly to in vitro infectivity assays i. e. cell culture (Pecková et al., 2016; Rousseau et al., 2018).

Table 1 presents some excystation applications within the environmental context. Assessing inactivation by excystation rate after exposure to ultraviolet radiation (UV) is noteworthy, as this disinfection method does not affect external morphology of the (oo)cysts i. e. walls or plasma membrane. UV causes damage to the nucleic acids of the cell (DNA/RNA) and may inhibit replication and transcription (Hijnen et al., 2006; Rochelle et al., 2005), which is not measureable by dye inclusion and/or exclusion techniques, for instance, and explains why most of the research on it requires infectivity assays (Karanis et al., 2003; Morita et al., 2002; Shin et al., 2001).

A study compared different protocols for induced excystation of *C. parvum* oocysts, which diverged in their respective stages of chemical pre-incubation, means of excystation or incubation time. Results indicated significant differences for the recovery of viable oocysts by modifying the pre-incubation procedures, but the other variables did not significantly differ in viability rate from a statistical point of view (Pecková et al., 2016).

A research comparing in vitro viability methods for *Cryptosporidium* spp. oocysts found that both molecular and staining techniques correlated well and properly discriminated viable from heat-killed oocysts, which was not achieved by excystation (Vande Burgt et al., 2018). The hierarchy and synergisms of stimuli that lead to excystation are not yet fully understood, as there is a variety of protocols presented in the literature (Einarsson et al., 2016; Pecková et al., 2016) and no standard procedure (Ortega-Pierres et al., 2018; Smith et al., 2005). Another limitation of the excystation method is that it requires a large number of purified (oo)cysts, which is not usually a realistic scenario in environmental samples (Rousseau et al., 2018). Although efforts to standardize in vitro excystation aiming at examining some of the biochemical triggers involved in this process date back decades (Hijjawi, 2010;

Table 1
Applications of excystation methods in environmental and sanitation research targeting protozoan cysts and oocysts.

Parasite	Treatment	Notes	Reference
C. parvum	Chlorination	The study endorses *C. parvum* resistant to conventional disinfectants and shows agreement with infectivity assay.	(Korich et al., 1990)
		Agreement with infectivity assay. Excystation overestimated oocyst viability following disinfection.	(Black et al., 1996)
		Comparison to fluorescent vital-dye uptake showed correlation (r-value = 0.91).	(Jenkins et al., 1997)
	Ozonation	Ozone was more effective to inactivate oocysts, but these were still considered resistant. Results showed agreement with infectivity assay.	(Korich et al., 1990)
		Agreement with infectivity assay. Excystation overestimated oocyst viability following disinfection. Agreement to fluorescent vital-dye uptake.	(Black et al., 1996)
		Excystation overestimated oocyst viability following disinfection. Results reaffirmed infectivity assays as the "gold standard". Low correlation to mouse infectivity (r-value = 0.23).	(Bukhari et al., 2000)
	Freezing Liming Contact with alum and ferric sulfate	Results reemphasized oocyst resistance and correlated to staining (r-value = 0.997).	(Robertson et al., 1992)
	Peroxidation	Excystation was carried out coupled to cell culture for in *vitro* infectivity.	(Weir et al., 2002)
		The study considers potential reasons why peroxidation renders oocysts nonviable.	(Kniel et al., 2004)
		Agreement with infectivity assay. Excystation overestimated oocyst viability following disinfection.	(Quilez et al., 2005)

(*continued on next page*)

Robertson et al., 1993), the methodological gap still exists and needs to be addressed.

Conventionally, *Giardia* trophozoites are detected by a number of features, including: median body shape and location, ventrolateral disc length, length of caudal flagella, and number and shape of nuclei. However, identifying trophozoites or cysts to species level is not possible simply by light microscopy (Monis et al., 2009) and staining techniques may assist detection of different stages of *Giardia*, as well as distinguishing them from other organisms and debris (Smith and Paget, 2007). Similarly, when undertaking excystation, *Cryptosporidium* oocysts expand a “suture” of their wall and each release four banana-shaped sporozoites, which often exhibit gliding motility (Bones et al., 2019). These modifications in morphology may be parameters in evaluating the organisms’ life cycle, thus applied research in automated morphometry (Liu et al., 2019), for instance, could shed light onto image-based viability assessment.

Table 1 (*continued*)

		Excystation was carried out coupled to cell culture for in *vitro* infectivity.	(Delling et al., 2017)
	UV (SODIS)	Agreement with infectivity assay. Excystation overestimated oocyst viability following disinfection. Matrix: Experimentally contaminated drinking water.	(McGuigan et al., 2006)
		Matrix: 30 NTU test water.	(Fontán-Sainz et al., 2012)
	UV	Excystation overestimated viability compared to animal infectivity.	(Morita et al., 2002)
		Authors mention that mechanisms for solar inactivation are not fully understood. Excystation levels did not decrease correspondently to infectivity in cell culture.	(King et al., 2008)
	Silver- and copper-embedded ceramic tablets	Agreement to fluorescent vital-dye uptake.	(Ehdaie et al., 2020)
G. duodenalis	Silver- and copper-embedded ceramic tablets	Agreement to fluorescent vital-dye uptake.	(Ehdaie et al., 2020)
G. muris	UV (SODIS)	Matrix: Experimentally contaminated drinking water. Agreement with infectivity assay.	(McGuigan et al., 2006)

Notes: All of the studies were performed with buffer solutions unless stated otherwise. SODIS = solar disinfection; UV = ultraviolet radiation.

Detection and counting may be carried out either in light or fluorescence microscopy, therefore it does not necessarily require expensive stains, although it is possible perform sample readings with fluorescent dyes for different cell structures, as well as using monoclonal antibodies (Wilke et al., 2018) for selective visualization. Released trophozoites and sporozoites may even be easy to spot in contrast to cysts and oocysts, as presented in a study that compared six excystation protocols, from which all released sporozoites were motile and therefore considered viable (Pecková et al., 2016). The motility feature is particularly interesting if considering automated counting based on image variation analysis (Kaakinen et al., 2014). Nevertheless, the simplicity in detection and its sensitivity rely on factors such as: the quantity of trophozoites and sporozoites present in the sample (Rousseau et al., 2018); the number of samples examined (Gutiérrez-Cisneros et al., 2011), the matrix under analysis; visualization media, i.e. which dye has been applied, as well as; if the sample was fresh (Smith and Paget, 2007) or dried and fixed.

3. Staining methods

Conventional staining has been widely applied to improve (oo)cyst visualization, particularly in parasitology research. Some common permanent dyes are MAF (modified acid-fast), DMSO-MAF (dimethyl sulfoxide-modified acid-fast), ACMV (analine carbol methyl violet), RIHS (regressive iron hematoxylin), Safranin, Leishman and Giemsa, applied in light microscopy of stool samples (Ahmed and Karanis, 2018). As for environmental samples, there are examples of modified Kinyoun's (MK) and modified Safranin Methylene Blue (SMB) light microscopy smears, performed in oil immersion field in light microscopy (Masangkay et al., 2020). These, however, do not lead to an estimation of viability, which is a role played by vital dyes. amongst the tests to infer viability through the application of vital dyes, some may be highlighted: the verification of enzyme-mediated reactions after substrate incorporation (staining "living" or supposedly viable cells) and/or dye inclusion, which will define the integrity of the cell membrane (staining "dead"/non-viable cells). Fluorescence reduction also has a part in evaluating cell damage, although it does not refer to dye inclusion. Due to the analytical difficulties mainly related to the visualization of cysts and oocysts, it is important to investigate different options of vital dyes.

3.1. Bright-field microscopy analysis

Erythrosin B and Trypan blue are versatile and comparatively low-cost vital dyes that have been used in many microorganisms, such as bacteria (Franke et al., 2020) and yeasts (Kwolek-Mirek and Zadrag-Tecza, 2014). Concerning protozoa, they have been applied in viability assays after cell culture targeting sporozoites, particularly (Emery et al., 2016).

Trypan blue is a precise example of a vital dye incorporated by cells with damaged membrane, which can be considered fragile or supposedly non-viable (Strober, 2015). Alongside to infectivity bioassays by faecal and intestinal parasitic counts, it has been applied for evaluating *in-vitro* viability of *Giardia* spp. and *Cryptosporidium* spp. infective stages, i. e. (oo)cysts., in disinfected samples (Zawawy et al., 2010). Similarly, Trypan blue has been used

to estimate the viability of *Cryptosporidium/Giardia* spp. (oo)cysts present in filtered water obtained after a conventional water treatment by flotation (Boni, 2016). The same study reported that though rapid and easy to perform, the test has analytical difficulties, due to the rapid loss of staining in both cysts and oocysts, as well as inherent difficulties in identifying them in bright-field microscopy, since the organisms might be easily mistaken by artefacts from the concentrated sample (Boni, 2016).

3.2. Fluorescence microscopy techniques

Fluorescent microscopy leads to a more clear visualization of the parasites, as they are expected to present high contrast against the background, when labelled with fluorescent stains. Particular examples of fluorescent staining in protozoa are displayed in Table 2, which emphasizes different matrices and contexts within the environmental and sanitation scene. Common fluorescent dyes are those based in ethidium and propidium, which have high affinity for nucleic acids, but are impermeable to the plasma membrane of the cells. Its incorporation occurs in a passive physicochemical manner, indicating that there is damage to the cell membrane, staining organisms that may be non-viable, weakened or dead (Netuschil et al., 2014).

Propidium iodide (PI) can be concomitantly applied to FITC (fluorescein-5-isothiocyanate) monoclonal antibodies, along with DAPI (4′,6-diamidino-2-phenylindole), a fluorescent blue stain that highlights the nuclei when bound to DNA. Aside to PI, this combination of stains is recommended for (oo)cyst detection in water and disinfected wastewater by Methods 1623.1 and 1693, respectively (USEPA, 2014, 2012). In these protocols, cysts and oocysts would be detected under FITC wavelengths of emission and excitation and visualized in apple green against a dark background; morphology would be confirmed by DAPI. Fig. 1 displays the expected set of images of a single non-viable cyst observed by these stains in conjunction to propidium iodide. The PI exclusion test consists on a simple additional staining process, which leads organisms considered non-viable to be observed in red under the PI-filter block. Concerning protozoa as target-organisms, PI has been widely applied in matrices such as treated wastewater (Kinyua et al., 2016), agricultural waste (Grit et al., 2012), water treatment residues (Sammarro Silva and Sabogal-Paz, 2020a) and drinking-water (Gómez-Couso et al., 2009). Fig. 2 displays the expected set for a viable *C. parvum* stained with both FITC monoclonal antibodies and PI. It is worth noting PI is a non-selective dye, therefore debris that contains genetic material might present staining, especially when environmental samples are considered.

As for green fluorescent dyes, which would be visualized under FITC filter (approximately 495 nm / 519 nm), there are options that can yield to fluorescence by different means, such as carboxyfluorescein diacetate (CD), CFDA-SE (carboxyfluorescein diacetate succinimidyl ester) and SYTOX® Green. These, however, are not selective stains and may not be of use when detection is carried out by immunofluorescence assays (IFA) with fluorophore-linked antibodies. IFA is the detection technique recommended by Methods 1693 and 1623.1 (USEPA, 2014, 2012), labelling (oo)cysts walls apple green, therefore interferences should be expected.

The mechanism for CFDA-SE and similar fluorescein molecules for green fluorescence bases on esterase activity, an intracellular enzyme. In this scene, viability is inferred by two means: the enzymatic activity, which is required to promote fluorescence when fluorescein acts as a substrate, and; the integrity of the cell membrane, necessary for the retention of fluorescent products within the cell. Because they are electrically neutral molecules, the esterase substrates freely diffuse into the cells. Once inside the cell, these non-fluorescent molecules are converted into fluorescent products that are then retained by organisms with intact plasma membranes. In contrast, substrates that are not hydrolysed rapidly leak from damaged membrane cells, even when there is still residual activity of esterase enzymes (Netuschil et al., 2014).

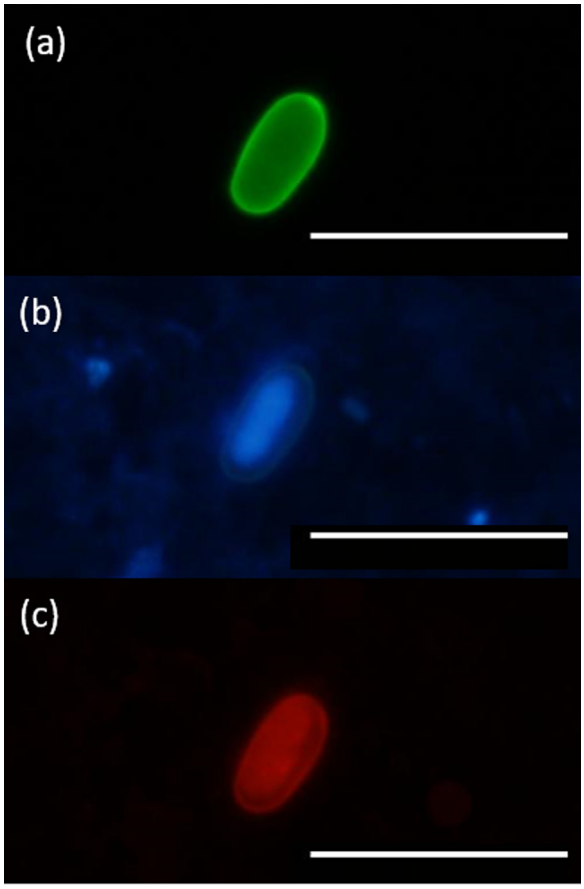

Fig. 1. Non-viable *Giardia duodenalis* cyst imaged by an Olympus® BX51 epifluorescence microscope under 800x magnification and labelled with (a) FITC; (b) DAPI; (c) propidium iodide, displaying red uptake. Scale br = 10 μm.

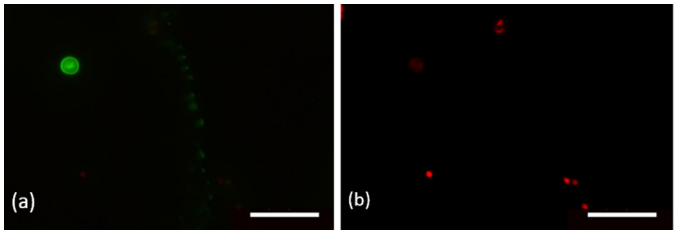

Fig. 2. Viable *C. parvum* oocyst recovered from filter backwash water samples imaged by an Olympus® BX51 epifluorescence microscope under 400x magnification and labelled with (a) FITC; (b) propidium iodide, lacking red-dye uptake by the oocyst. Scale bar = 20 μm.

CD exhibits green fluorescence in viable cells and has played a role in environmental analysis. This dye has been applied to verify the viability of bacteria in biofilms (Netuschil et al., 2014) and algae (MacIntyre and Cullen, 2016), including ballast water treatment systems with ultraviolet disinfection (Sun and Blatchley, 2017). However, a study in dyed protozoa under stress conditions (Santos et al., 2015) reported that only damaged, dead and dying (oo)cysts were able to interact with CFDA-SE, expected to passively enter the cytoplasm and yield to fluorescence by esterase metabolism.

In order to overcome limitations in stain-based techniques, the combination of vital dyes can be applied to simultaneously

Table 2
– Viability assessment through fluorescent staining of protozoan cysts and oocysts in different matrices within environmental and sanitation contexts.

Dye	Parasite	Matrix	Treatment / environmental condition	Reference
CFDA-SE	*C. parvum*	Buffer solution	Heating and UV	(Santos et al., 2015) *
	G. duodenalis		Heating and UV	(Santos et al., 2015) *
PI	*C. parvum*	Buffer solution	Peroxidation with H_2O_2-based disinfectants	(Quilez et al., 2005)*
			Photocatalysis (TiO_2/H_2O_2)	(Abeledo-Lameiro et al., 2017)
		Experimentally contaminated influent of anaerobic digester for swine waste treatment	Anaerobic digestion	(Kinyua et al., 2016)
		Test waters with different turbidities (5, 10 and 300 NTU)	UV (SODIS)	(Gómez-Couso et al., 2009)*
	G. duodenalis	Experimentally contaminated drinking water	UV (SODIS and SOPAS)	(Mtapuri-Zinyowera et al., 2009)
		Agricultural waste (cattle slurry)	Environmental conditions over time (storage up to 90 days)	(Grit et al., 2012)*
		Experimentally contaminated feed of anaerobic digester for swine wastewater treatment	Anaerobic digestion	(Kinyua et al., 2016)
PI + DAPI	*C. parvum*	Buffer solution	Ozonation	(Korich et al., 1990)*
			Ozonation	(Black et al., 1996)*
			Ozonation	(Bukhari et al., 2000)
			Freezing Liming Contact with alum and ferric sulfate	(Robertson et al., 1992)
			Chlorination	(Black et al., 1996)* (Jenkins et al., 1997)
			Ozonation	(Black et al., 1996)*
			Ozonation	(Bukhari et al., 2000)
		Experimentally contaminated drinking water	UV (SODIS)	(McGuigan et al., 2006)*
		Buffer solution	Silver- and copper-embedded ceramic tablets	(Ehdaie et al., 2020)
	G. duodenalis	Buffer solution	Silver- and copper-embedded ceramic tablets	(Ehdaie et al., 2020)
	G. muris	Experimentally contaminated drinking water	UV (SODIS)	(McGuigan et al., 2006)*
PI + IFA	*C. parvum*	Distilled water	Photo-Fenton	(Abeledo-Lameiro et al., 2019)
		Real and filtered water from a water treatment plant	Sonication	(Abeledo-Lameiro et al., 2018)
	Cryptosporidium spp.	Raw and treated water from a WTP	Conventional water treatment	(Ramo et al., 2017b)
		Simulated filter backwash water	Ozonation	(Silva and Sabogal-Paz, 2020)

(*continued on next page*)

Table 2 (*continued*)

		Simulated residue from flotation	Liming	
	Giardia spp.	Raw and treated water from a WTP	Conventional water treatment	(Ramo et al., 2017b)
		Simulated filter backwash water	Ozonation	(Silva and Sabogal-Paz, 2020)
		Simulated residue from flotation	Liming	
SYTO™ 9	*C. parvum*	Buffer solution	Ozonation	(Bukhari et al., 2000)
SYTO™ 9 + PI and DAPI + PI	*Cryptosporidium* spp. *Giardia* spp.	Wastewater	Chlorination and UV	(Adeyemo et al., 2019)

Notes: References indicated with (*) stand for results that were compared infectivity assays and agreed to them. CFDA-SE = carboxyfluorescein diacetate succinimidyl ester; DAPI = 4′,6-diamidino-2-phenylindole; H_2O_2 = hydrogen eroxide; IFA = immunofluorescence assay; NTU = nephelometric turbidity units; PI = propidium iodide; SODIS = solar disinfection; SOPAS = solar pasteurization; UV = ultraviolet; WTP = water treatment plant.

analyse exclusion/inclusion methods, in which one non-fluorescent dye is incorporated and metabolized to fluoresce in living cells, and another is embedded by cells with damaged membranes (Rousseau et al., 2018). This effect is expected by some commercial kits that suggest the simultaneous visualization of cells considered "live" and "dead", through microscopy or flow cytometry. Some down sides of this methodology are that these kits are expensive, as well as the fluorescence quantification equipment (Franke et al., 2020).

Research regarding simultaneous viability assessment associated to esterase activity and membrane damage refers mostly to bacteria (Boulos et al., 1999; Hu et al., 2017; Netuschil et al., 2014), helminth eggs (Karkashan et al., 2014), and helminth larvae (Sena-Lopes et al., 2020) in biofilms or the environment. Although these investigation efforts have not yet been widely directed towards protozoa, there is a study of simultaneous staining in *G. duodenalis* and *C. parvum* (oo)cysts, which reported low visualization of fluorescent esterase products in viable parasites, when not coupled to monoclonal antibodies for detection, whilst these were interferential to viability assessment (Sammarro Silva and Sabogal-Paz, 2020b). In addition, these assays do not always provide a discrete live or dead category, as intermediate stages are possible (Berney et al., 2007; Davey, 2011; Davey and Hexley, 2011).

On the topic of sanitation, concurrent staining by SYBR® Green and PI have been used to evaluate disinfection efficiency by bacterial inactivation (Bigoni et al., 2014) in a solar-based water treatment. Nevertheless, this combination is not selective towards active cells, as SYBR® Green enters all cells and binds to double-stranded DNA. The expected effect in protozoan (oo)cysts would be the analogous as the standard green visualization for detection under FITC as in IFA, coupled with PI inclusion when there is plasma membrane damage evaluation. Therefore, this scenario does not represent a simultaneous live/dead analysis, as only PI refers to viability *per se*.

There is an example, nonetheless, of a simultaneous viability assessment in protozoa, using an opposite approach to refer to viable and non-viable cells, in terms of the dyes in question. The study (Santos et al., 2015) combined the use of C12-resazurin and SYTOX® Green from a live/dead commercial kit and applied these probes in *Cryptosporidium/Giardia* spp. (oo)cysts in different conditions. In active cells, C12-resazurin is reduced to red C12-resorufin, while SYTOX® Green is only incorporated by compromised membranes and stains the nucleic acid of apoptotic and dead cells only, so there are two viability "parameters" analysed simultaneously, although different from those previously reported. If both stains are applied, cells with normal physiological activity get reddish-orange, while damaged cells yield to green fluorescence of their nuclei. Though, the research (Santos et al., 2015) emphasized the inability of fresh and infectious *C. parvum* and *G. duodenalis* to take up the referred stains but aged and dead cysts were double-marked when C12-resazurin and SYTOX® Green were applied. These results may be associated to the (oo)cyst morphology that also relates to their resistance, which make them such important target organisms. Therefore, although there are incongruences and some misleading effects in fluorescence staining (concurrent or not), further research contemplating protozoan (oo)cysts in different matrices and treatment conditions is encouraged.

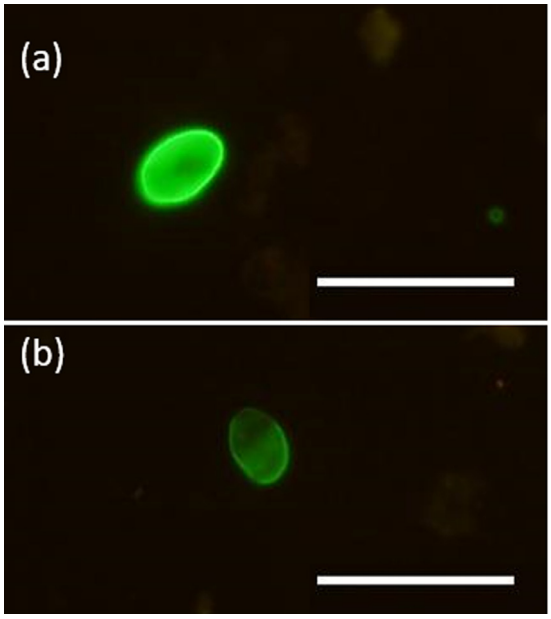

Fig. 3. *Giardia duodenalis* cysts imaged by an Olympus® BX51 epifluorescence microscope under 400x magnification and labelled with FITC by immunofluorescence assay, displaying (a) regular fluorescence; (b) altered fluorescence. Scale bar = 10 μm.

3.3. Fluorescence reduction assessment

Observing fluorescence decrease and wall damage has been reported as an alternative for estimating *Giardia* spp. cysts viability, particularly in effluents of advanced oxidative processes (AOPs) (Guimarães et al., 2014; Kondo Nakada et al., 2018). The assessment is based on the reduction in the expected apple-green fluorescence of the cysts and/or shape alterations after an immunofluorescence reaction (IFA), as shown in Fig. 3. This points fluorescence reduction as non-effective to assess viability after UV treat-

ments, for instance, as reported by Medeiros et al. (2020), who did not find significant alterations in fluorescence after UV disinfection of wastewater.

The principle of this assay relates to wall integrity, as IFA directly depends on the adherence of a monoclonal antibody to the epitopes of a cyst. The antibody is attached to FITC, a fluorescent marker that will enhance *Giardia* spp. or *Cryptosporidium* spp. cysts against the background. As aforementioned, this procedure is often applied for detection, such as indicated by Methods 1623.1 and 1693 (USEPA, 2014, 2012). Hence, fluorescence reduction assessment is an alternative to couple viability estimation to (oo)cyst detection within a single examination.

4. Complementary analyses

Employing more than one microscopy method may improve *Cryptosporidium* and *Giardia* detection from matrices such as surface water, groundwater with sediments, and substrate-associated biofilms (Masangkay et al., 2020). Accordingly, it may also improve viability assessment, as combinations of techniques have been explored (McGuigan et al., 2006) to provide more reliable results in control measures.

Alterations in morphology and physical properties may indicate viability loss at the single parasite level (Rousseau et al., 2018). These may be observed through sophisticated microscopy techniques (e.g. scanning electron microscopy) or inferred by non-invasive methods. The latter points to electrorotation, which is a procedure based on electric fields to which microorganisms are subjected. The speed and direction of rotation will vary according to conductivity and permittivity of the organism's constituent components e. g. wall, plasma membrane and cytoplasm (Dalton et al., 2004). Electrorotation viability assessment is carried out by analysing a rotation spectra and the probes may be coupled to large field microscopy imaging in order to assess multiple cells (Dalton et al., 2004; Dalton et al., 2001). Although it is a rapid analysis that allows real time measure and has correlated to PI exclusion in (oo)cysts, electrorotation data lacks information relative to infectivity assessment and influence of different matrices to results (Dalton et al., 2004, 2001; Rousseau et al., 2018).

Structural damage caused by ozone in *G. duodenalis* cysts have been studied by fluorescence staining in flow cytometry coupled to scanning electron microscopy (SEM) (Widmer et al., 2002). In this assessment, experienced microscopists, blinded to the sample identity, ranked multiple micrographs according to the extent of the structural alteration found in cysts. Similarly, fluorescence reduction has been applied concomitantly to SEM to assess viability in *G. duodenalis* cysts after a H_2O_2/UV treatment (Guimarães et al., 2015). SEM imaging has also correlated to fluorescein diacetate and propidium iodide staining to evaluate degradation of *G. duodenalis* cysts in mixed swine and human wastes under field conditions (Deng and Cliver, 1992). As of *C. parvum*, a study on polymer adhesion explored the biding of viable and non-viable oocysts on selected polymers and the technique allowed exploring different morphology features visualized by SEM (Wu et al., 2012).

Simple complimentary analysis are also endorsed in microscopy. A study in automated detection used an immunofluorescence assay to generate binary images and identify cysts and oocyst by their roundness (Badsha et al., 2013). Authors assessed viability by counting nuclei stained with DAPI. This approach may lead to possible correspondences amongst technologies (e.g. scales of fluorescence reduction, binary images from PI uptake etc.) and encourage further research in patter recognition and automation of image-based viability assessment.

Although they are not microscopy-based, there is a rising popularity in molecular techniques to assess viability. Rousseau et al. (2018) describes applications of RNA- and DNA-based methods in foodborne and waterborne stages of pathogenic protozoa and encourages applying these methods for determining initial levels of contamination.

5. General comparison

Table 3 displays an overall comparison of the viability methods described throughout this review, framing topics that might act as either advantages or limitations. The different aspects of each technique must be considered when selecting the most adequate method for each situation, as laboratories present different infrastructure conditions, budgets, and specialized personnel. In addition, environmental samples may vary in characteristics. The matrix aspect is particularly relevant, since debris could impair bright-field microscopy visualization, but also on account of the (oo)cyst recovery process. The latter has been reported as affecting propidium iodide uptake in organisms recovered from complex matrices (Sammarro Silva and Sabogal-Paz, 2020a).

There are timely studies covering (oo)cyst detection and the role of recovery efficiency (Efstratiou et al., 2017a; Ongerth, 2013), where authors explore the pressing need of standardization for monitoring. Nonetheless, as pointed by Efstratiou et al. (2017a), there is a faulty concept regarding the idea of a single protocol to "fit all" sanitation needs. We believe the same applies to viability assessment, and the prospect of a "universal" protocol seems unrealistic. However, this should not be regarded as an issue, as long as the chosen technique is consistent to the goal and there is transparency towards the research outcomes.

6. Concluding remarks

Excystation methods provide an active estimation of viability and may be of use particularly when this parameter does not relate to physical damage within walls or membranes of the (oo)cysts, as in UV treatment. Nevertheless, there is an operational limitation as these analyses are not easy to standardize to a routine laboratory test. Excystation also requires incubation and specific conditions to each parasite, as well as clean suspensions and a high number of organisms. These matters, however, should not be taken as obstructing research, but as encouraging prospects to more focused goals in parasitology, so that sanitation and environmental monitoring, as well as other fields, can properly uphold to.

Vital staining presents limitations that should not be disregarded, particularly concerning overestimation and false-positives. When carried out in fluorescence, it requires infrastructure that may be deterrent to some laboratories. However, its simplicity in screening and potential to investigate multiple parasites are advantages for routine analysis and pattern recognition, which may explain the broad application of vital staining in protozoan viability assessment in the environmental field. We believe conventional stains as in propidium iodide, that can be applied coupled to commercial kits for (oo)cyst detection should be satisfactory to sanitation companies when carrying out regular tests, for instance. Nonetheless, innovative and simple approaches as in single-panel visualization, as well as fluorescence reduction are open subjects for research and should be regarded by scientists in a matter of quantitatively describing correlation to infectivity. In addition, providing images as supplementary data would be of great use to the science community. Laboratories equipped with powerful imaging set ups could also focus on building protocoled datasets that could support collaborative research in fields as in geometric morphometric or automated data classification by algorithms.

As for exploratory tests, we also recommend backing up one technique with another and investigating possible correlations, as well as potential for complementary analyses. When these are carried out, it is crucial that limitations are covered and applica-

Table 3
– General features of microscopy-based viability assessment methods.

Advantages / limitations	Viability assessment method						
	Induced excystation				Vital staining		
	BF	Fluorescence[3]	Coupled to IFA[3]	Coupled to cell culture	BF	Fluorescence	Coupled to IFA
Application on complex matrices	(-)	(-)	(-)	(-)	(-)	(-)	(+)
Considers damage to the DNA level	(+)	(+)	(+)	(+)	(-)	(-)	(-)
Agreement with bioassays[1]	(+)[a, b, c]	(+)	(+)	(+/-)[4, d]	NA	(+/-)[a, c, g]	(+/-)[c]
Correlation with PI exclusion	(+)[b, c]	(+)	(+)	(+/-)[4, d]	NA	(+)[b, g]	(+)
Non-Expensive	(+)	(+)	(-)	(+)	(+)	(+)	(-)
Rapid	(+)	(+)	(-)	(-)	(+)	(+)	(+)
Reliable assessment of inactivation efficacy[2]	(-)	(-)	(-)	(+/-)[e,f]	(-)	(-)	(-)
Requires a darkened room and epifluorescence microscope	(-)	(+)	(+)	(-)	(-)	(+)	(+)
Requires a single (oo)cyst	(-)	(-)	(-)	(-)	(+)	(+)	(+)
Requires incubation (hours)	(+)	(+)	(+)	(+)	(-)	(-)	(-)
Requires purified (oo)cysts	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Standardized methods / easy to standardize	(-)	(-)	(-)	(-)	(+)	(+)	(+)
Targets multiple parasites	(-)	(-)	(-)	(-)	(+)	(+)	(+)

Notes: [1]Although some studies report agreement with infectivity assays, they also mention overestimation of viability.
[2] Based on viability relative to infectivity.
[3] These will only differ from BF methods in the visualization set up, therefore some advantages and limitations were inferred from fluorescence staining and IFA techniques in this regard, whilst general excystation properties remain.
[4] This refers to in vitro infectivity, thus the understanding of correlation will depend on either excystation itself or cell invasion compared to bioassays or PI exclusion.
[a](Korich et al., 1990).
[b] (Black et al., 1996).
[c] (McGuigan et al., 2006).
[d] (King et al., 2008).
[e] (Rousseau et al., 2018).
[f] (Karanis, 2018).
[g] (Bukhari et al., 2000); BF = brightfield; IFA = immunofluorescence assay; NA = no data available/ PI = propidium iodide; (+) yes; (-) no; (+/-) data varies.

tions are clear, so that endpoint professionals may properly decide for a technique. When doing so, particularities associated to the microorganism, the matrix, laboratory infrastructure and goal of the research must be considered to determine the most suitable method for each scenario, from routine analysis to the development of lab-on-chip devices.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by Global Challenges Research Fund (GCRF) UK Research and Innovation (SAFEWATER; EPSRC Grant Reference EP/P032427/1) and by the Coordination for the Improvement of Higher Education Personnel (CAPES-Brazil, Financial Code: 001) for the PhD scholarship awarded to Kamila Jessie Sammarro Silva.

References

Abeledo-Lameiro, M.J., Ares-Mazás, E., Goméz-Couso, H., 2018. Use of ultrasound irradiation to inactivate Cryptosporidium parvum oocysts in effluents from municipal wastewater treatment plants. Ultrason. Sonochem. 48, 118–126. doi:10.1016/j.ultsonch.2018.05.013.

Abeledo-Lameiro, M.J., Polo-López, M.I., Ares-Mazás, E., Gómez-Couso, H., 2019. Inactivation of the waterborne pathogen Cryptosporidium parvum by photo-Fenton process under natural solar conditions. Appl. Catal. B Environ. 253, 341–347. doi:10.1016/j.apcatb.2019.04.049.

Abeledo-Lameiro, M.J., Reboredo-Fernández, A., Polo-López, M.I., Fernández-Ibáñez, P., Ares-Mazás, E., Gómez-Couso, H., 2017. Photocatalytic inactivation of the waterborne protozoan parasite Cryptosporidium parvum using TiO2/H2O2 under simulated and natural solar conditions. Catal. Today 280, 132–138. doi:10.1016/j.cattod.2016.05.046.

Adeyemo, F.E., Singh, G., Reddy, P., Bux, F., Stenström, T.A., 2019. Efficiency of chlorine and UV in the inactivation of Cryptosporidium and Giardia in wastewater. PLoS ONE 14, 1–22. doi:10.1371/journal.pone.0216040.

Ahmed, S.A., Karanis, P., 2018. Comparison of current methods used to detect Cryptosporidium (oo)cysts in stools. Int. J. Hyg. Environ. Health 221, 743–763. doi:10.1016/j.ijheh.2018.04.006.

Badsha, S., Mokhtar, N., Arof, H., Lim, Y.A.L., Mubin, M., Ibrahim, Z., 2013. Automatic Cryptosporidium and Giardia viability detection in treated water. EURASIP J. Image Video Process. 1–10. doi:10.1186/1687-5281-2013-56, 2013.

Berney, M., Hammes, F., Bosshard, F., Weilenmann, H.-.U., Egli, T., 2007. Assessment and interpretation of bacterial viability by using the LIVE/DEAD baclight kit in combination with flow cytometry. Appl. Environ. Microbiol. 73, 3283–3290. doi:10.1128/AEM.02750-06.

Bigoni, R., Kötzsch, S., Sorlini, S., Egli, T., 2014. Solar water disinfection by a parabolic trough concentrator (PTC): flow-cytometric analysis of bacterial inactivation. J. Clean. Prod. 67, 62–71. doi:10.1016/j.jclepro.2013.12.014.

Black, E.K., Finch, G.R., Thaghi-Kilani, R., Belosevic, M., 1996. Comparison of viability assays for Cryptosporidium parvum oocysts after disinfection. FEMS Microbiol. Lett. 135, 187–189.

Bones, A.J., Jossé, L., More, C., Miller, C.N., Michaelis, M., Tsaousis, A.D., 2019. Past and future trends of Cryptosporidium in vitro research. Exp. Parasitol. 196, 28–37. doi:10.1016/j.exppara.2018.12.001.

Boni, D.M., 2016. Viability Assessment of Giardia spp. Cysts and Cryptosporidium parvum Oocysts in Filtered Water Obtained After Conventional Treatment With Flotation and Ozonation (Avaliação Da Viabilidade De Cistos De Giardia spp. e Oocistos De Cryptosporidium parvum Em água filtrada Obtida Após Tratamento Convencional Com Flotação e Ozonização). University of São Paulo doi:10.11606/D.18.2017.tde-28032017-095459.

Boucher, S.E.M., Gillin, F.D., 1990. Excystation of in vitro-derived Giardia lamblia cysts. Infect. Immun. 58, 3516–3522.

Boulos, L., Prévost, M., Barbeau, B., Coallier, J., Desjardins, R., 1999. LIVE/DEAD® BacLight: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J. Microbiol. Methods 37, 103–104. doi:10.1016/s0167-7012(99)00048-2.

Boyer, D.G., Kuczynska, E., Fayer, R., 2009. Transport, fate, and infectivity of Cryptosporidium parvum oocysts released from manure and leached through macroporous soil. Environ. Geol. 58, 1011–1019. doi:10.1007/s00254-008-1580-x.

Brescia, C.C., Griffin, S.M., Ware, M.W., Varughese, E.A., Egorov, A.I., Villegas, E.N., 2009. Cryptosporidium propidium monoazide-PCR, a molecular biology-based technique for genotyping of viable Cryptosporidium oocysts. Appl. Environ. Microbiol. 75, 6856–6863. doi:10.1128/AEM.00540-09.

Bukhari, Z., Marshall, M.M., Korich, D.G., Fricker, C.R., Smith, H.V., Rosen, J., Clancy, J.L., 2000. Comparison of Cryptosporidium parvum viability and infectivity assays following ozone treatment of oocysts. Appl. Environ. Microbiol. 66, 2972–2980. doi:10.1128/AEM.66.7.2972-2980.2000.

Cacciò, S.M., Sprong, H., 2011. Epidemiology of Giardiasis in Humans, pp. 17–28, Giardia. doi:10.1007/978-3-7091-0198-8_2.

Cacciò, S.M., Widmer, G., 2014. Cryptosporidium: Parasite and disease, Cryptosporidium: Parasite and Disease doi:10.1007/978-3-7091-1562-6.

Cama, V.A., Mathison, B.A., 2015. Infections by intestinal Coccidia and Giardia duodenalis. Clin. Lab. Med. 35, 423–444. doi:10.1016/j.cll.2015.02.010.

Carey, C.M., Lee, H., Trevors, J.T., 2004. Biology, persistence and detection of Cryptosporidium parvum and Cryptosporidium hominis oocyst. Water Res. 38, 818–862. doi:10.1016/j.watres.2003.10.012.

Castro-Hermida, J.A., García-Presedo, I., Almeida, A., González-Warleta, M., Correia Da Costa, J.M., Mezo, M., 2009. Detection of Cryptosporidium spp. and Giardia

duodenalis in surface water: a health risk for humans and animals. Water Res. 43, 4133–4142. doi:10.1016/j.watres.2009.06.020.
Chique, C., Hynds, P.D., Andrade, L., Burke, L., Morris, D., Ryan, M.P., O'Dwyer, J., 2020. Cryptosporidium spp. in groundwater supplies intended for human consumption – a descriptive review of global prevalence, risk factors and knowledge gaps. Water Res. 176, 115726. doi:10.1016/j.watres.2020.115726.
Chuah, C.J., Mukhaidin, N., Choy, S.H., Smith, G.J.D., Mendenhall, I.H., Lim, Y.A.L., Ziegler, A.D., 2016. Prevalence of Cryptosporidium and Giardia in the water resources of the Kuang river catchment. Northern Thailand. Sci. Total Environ. 562, 701–713. doi:10.1016/j.scitotenv.2016.03.247.
Dalton, C., Goater, A.D., Burt, J.P.H., Smith, H.V., 2004. Analysis of parasites by electrorotation. J. Appl. Microbiol. 96, 24–32. doi:10.1046/j.1365-2672.2003.02113.x.
Dalton, C., Goater, A.D., Pethig, R., Smith, H.V., 2001. Viability of Giardia intestinalis cysts and viability and sporulation state of Cyclospora cayetanensis oocysts determined by electrorotation. Appl. Environ. Microbiol. 67, 586–590. doi:10.1128/AEM.67.2.586-590.2001.
Davey, H.M., 2011. Life, death, and in-between: meanings and methods in microbiology. Appl. Environ. Microbiol. 77, 5571–5576. doi:10.1128/AEM.00744-11.
Davey, H.M., Hexley, P., 2011. Red but not dead? Membranes of stressed Saccharomyces cerevisiae are permeable to propidium iodide. Environ. Microbiol. 13, 163–171. doi:10.1111/j.1462-2920.2010.02317.x.
de Araújo, R.S., Aguiar, B., Dropa, M., Razzolini, M.T.P., Sato, M.I.Z., de Souza Lauretto, M., Galvani, A.T., Padula, J.A., Matté, G.R., Matté, M.H., 2018. Detection and molecular characterization of Cryptosporidium species and Giardia assemblages in two watersheds in the metropolitan region of São Paulo. Brazil. Environ. Sci. Pollut. Res. 25, 15191–15203. doi:10.1007/s11356-018-1620-3.
Delling, C., Lendner, M., Müller, U., Daugschies, A., 2017. Improvement of in vitro evaluation of chemical disinfectants for efficacy on Cryptosporidium parvum oocysts. Vet. Parasitol. 245, 5–13. doi:10.1016/j.vetpar.2017.07.018.
DuPont, H.L., Chappell, C.L., Sterling, C.R., Okhuysen, P.C., Rose, J.B., Jakubowski, W., 1995. The infectivity of Cryptosporidium parvum in healthy volunteers. N. Engl. J. Med. 332, 855–859. doi:10.1056/NEJM199503303321304.
Efstratiou, A., Ongerth, J., Karanis, P., 2017a. Evolution of monitoring for Giardia and Cryptosporidium in water. Water Res 123, 96–112. doi:10.1016/j.watres.2017.06.042.
Efstratiou, A., Ongerth, J.E., Karanis, P., 2017b. Waterborne transmission of protozoan parasites: review of worldwide outbreaks - an update 2011–2016. Water Res. 114, 14–22. doi:10.1016/j.watres.2017.01.036.
Ehdaie, B., Su, Y.H., Swami, N.S., Smith, J.A., 2020. Protozoa and virus disinfection by silver- and copper-embedded ceramic tablets for water purification. J. Environ. Eng. (United States) 146, 1–10. doi:10.1061/(ASCE)EE.1943-7870.0001664.
Einarsson, E., Troell, K., Hoeppner, M.P., Grabherr, M., Ribacke, U., Svärd, S.G., 2016. Coordinated changes in gene expression throughout encystation of Giardia intestinalis. PLoS Negl. Trop. Dis. 10, 1–22. doi:10.1371/journal.pntd.0004571.
Emery, S.J., Mirzaei, M., Vuong, D., Pascovici, D., Chick, J.M., Lacey, E., Haynes, P.A., 2016. Induction of virulence factors in Giardia duodenalis independent of host attachment. Sci. Rep. 6, 20765. doi:10.1038/srep20765.
Fernandes, L.N., De Souza, P.P., De Araújo, R.S., Razzolini, M.T.P., Soares, R.M., Sato, M.I.Z., Hachich, E.M., Cutolo, S.A., Matté, G.R., Matté, M.H., 2011. Detection of assemblages A and B of Giardia duodenalis in water and sewage from São Paulo state. Brazil. J. Water Health 9, 361–367. doi:10.2166/wh.2011.098.
Fontán-Sainz, M., Gómez-Couso, H., Fernández-Ibáñez, P., Ares-Mazás, E., 2012. Evaluation of the solar water disinfection process (SODIS) against Cryptosporidium parvum using a 25-L static solar reactor fitted with a compound parabolic collector (CPC). Am. J. Trop. Med. Hyg. 86, 223–228. doi:10.4269/ajtmh.2012.11-0325.
Franke, J.D., Braverman, A.L., Cunningham, A.M., Eberhard, E.E., Perry, G.A., 2020. Erythrosin B: a versatile colorimetric and fluorescent vital dye for bacteria. BioTechniques 68, 7–13. doi:10.2144/btn-2019-0066.
Geurden, T., Olson, M., 2011. Giardia in pets and farm animals, and their zoonotic potential. In: Giardia. Springer, Vienna, Vienna, pp. 71–92. doi:10.1007/978-3-7091-0198-8_4.
Gómez-Couso, H., Fontán-Sainz, M., McGuigan, K.G., Ares-Mazás, E., 2009. Effect of the radiation intensity, water turbidity and exposure time on the survival of Cryptosporidium during simulated solar disinfection of drinking water. Acta Trop 112, 43–48. doi:10.1016/j.actatropica.2009.06.004.
Graczyk, T.K., Kacprzak, M., Neczaj, E., Tamang, L., Graczyk, H., Lucy, F.E., Girouard, A.S., 2008. Occurrence of Cryptosporidium and Giardia in sewage sludge and solid waste landfill leachate and quantitative comparative analysis of sanitization treatments on pathogen inactivation. Environ. Res. 106, 27–33. doi:10.1016/j.envres.2007.05.005.
Grit, G.H., Bénéré, E., Ehsan, A., De Wilde, N., Claerebout, E., Vercruysse, J., Maes, L., Geurden, T., 2012. Giardia duodenalis cyst survival in cattle slurry. Vet. Parasitol. 184, 330–334. doi:10.1016/j.vetpar.2011.08.021.
Guimarães, J.R., Franco, R.M.B., Guadagnini, R.A., Santos, L.U.dos, 2014. Giardia duodenalis : number and fluorescence reduction caused by the advanced oxidation process (H 2 O 2 /UV). Int. Sch. Res. Not. 1–7. doi:10.1155/2014/525719, 2014.
Guimarães, J.R., Santos, L.U.D., Franco, R.M.B., Guadagnini, R.A., 2015. Inactivation of Giardia duodenalis cysts by peroxidation and peroxidation assisted by ultraviolet radiation (Inativação de cistos de Giardia duodenalis por peroxidação e peroxidação assistida por radiação ultravioleta). Eng. Sanit. e Ambient. 20, 159–164. doi:10.1590/S1413-41522015020000098360.
Gutiérrez-Cisneros, M.J., Martínez-Ruiz, R., Subirats, M., Merino, F.J., Millán, R., Fuentes, I., 2011. Assessment of two commercially available immunochromatographic assays for a rapid diagnosis of Giardia duodenalis and Cryptosporidium spp. in human faecal specimens (Evaluación de dos métodos inmunocromatográficos comerciales para el diagnóstico rápido de Giardia duodenalis y Cryptosporidium spp. en muestras de heces). Enferm. Infecc. Microbiol. Clin. 29, 201–203. doi:10.1016/j.eimc.2010.09.005.
Hijjawi, Nawal, 2010. Cryptosporidium: New developments in cell culture. Experimental Parasitology 124 (1), 54–60. doi:10.1016/j.exppara.2009.05.015.
Hijnen, W.A.M., Beerendonk, E.F., Medema, G.J., 2006. Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: a review. Water Res 40, 3–22. doi:10.1016/j.watres.2005.10.030.
Hu, W., Murata, K., Zhang, D., 2017. Applicability of LIVE/DEAD BacLight stain with glutaraldehyde fixation for the measurement of bacterial abundance and viability in rainwater. J. Environ. Sci. (China) 51, 202–213. doi:10.1016/j.jes.2016.05.030.
Jenkins, M., Anguish, L.J., Bowman, D.D., Walker, M.J., Ghiorse, W.C., 1997. Assessment of a dye permeability assay for determination of inactivation rates of Cryptosporidium oocysts. Appl. Environ. Microbiol. 63, 3844–3850.
Kaakinen, M., Huttunen, S., Paavolainen, L., Marjomaki, V., Heikkila, J., Eklund, L., 2014. Automatic detection and analysis of cell motility in phase-contrast time-lapse images using a combination of maximally stable extremal regions and Kalman filter approaches. J. Microsc. 253, 65–78. doi:10.1111/jmi.12098.
Karanis, P., 2018. The truth about in vitro culture of Cryptosporidium species. Parasitology 145, 855–864. doi:10.1017/S0031182017001937.
Karanis, P., Schoenen, D., Hoyer, O., 2003. Inactivation of Cryptosporidium parvum oocysts by low pressure UV-light. Cryptosporidium Mol. Dis. 261–263. doi:10.1016/B978-044451351-9/50036-7.
Karkashan, A., Khallaf, B., Morris, J., Thurbon, N., Rouch, D., Smith, S.R., Deighton, M., 2014. 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. 68, 533–544. doi:10.1016/j.watres.2014.10.003.
Kifleyohannes, T., Robertson, L.J., 2020. Preliminary insights regarding water as a transmission vehicle for Cryptosporidium and Giardia in Tigray. Ethiopia. Food Waterborne Parasitol. 19. doi:10.1016/j.fawpar.2020.e00073.
King, B.J., Hoefel, D., Daminato, D.P., Fanok, S., Monis, P.T., 2008. Solar UV reduces Cryptosporidium parvum oocyst infectivity in environmental waters. J. Appl. Microbiol. 104, 1311–1323. doi:10.1111/j.1365-2672.2007.03658.x.
Kinyua, M.N., Trimmer, J., Izurieta, R., Cunningham, J., Ergas, S.J., 2016. Viability and fate of Cryptosporidium parvum and Giardia lamblia in tubular anaerobic digesters. Sci. Total Environ. 554–555, 167–177. doi:10.1016/j.scitotenv.2016.02.170.
Kniel, K.E., Sumner, S.S., Pierson, M.D., Zajac, A.M., Hackney, C.R., Fayer, R., Lindsay, D.S., 2004. Effect of hydrogen peroxide and other protease inhibitors on Cryptosporidium parvum excystation and in vitro development. J. Parasitol. 90, 885–888. doi:10.1645/ge-203r1.
Koehler, A.V., Jex, A.R., Haydon, S.R., Stevens, M.A., Gasser, R.B., 2014. Giardia/giardiasis - a perspective on diagnostic and analytical tools. Biotechnol. Adv. 32, 280–289. doi:10.1016/j.biotechadv.2013.10.009.
Kondo Nakada, L.Y., Bueno Franco, R.M., da Silva Fiuza, V.R., Urbano dos Santos, L., Branco, N., Guimarães, J.R., 2018. Pre-ozonation of source water: assessment of efficacy against Giardia duodenalis cysts and effects on natural organic matter. Chemosphere 214, 764–770. doi:10.1016/J.CHEMOSPHERE.2018.09.164.
Korich, D.G., Mead, J.R., Madore, M.S., Sinclair, N.A., Sterling, C.R., 1990. Effects of ozone, chlorine dioxide, chlorine, and monochloramine on Cryptosporidium parvum oocyst viability. Appl. Environ. Microbiol. 56, 1423–1428.
Koydemir, H.C., Gorocs, Z., Tseng, D., Cortazar, B., Feng, S., Chan, R.Y.L., Burbano, J., McLeod, E., Ozcan, A., 2015. Rapid imaging, detection and quantification of Giardia lamblia cysts using mobile-phone based fluorescent microscopy and machine learning. Lab Chip 15, 1284–1293. doi:10.1039/C4LC01358A.
Kumar, T., Azlan, M., Majid, A., Onichandran, S., Jaturas, N., Andiappan, H., Salibay, C.C., Tabo, H.A.L., Tabo, N., Dungca, J.Z., Tangpong, J., Phiriyasamith, S., Yuttayong, B., Polseela, R., Do, B.N., Sawangjaroen, N., 2016. Presence of Cryptosporidium parvum and Giardia lamblia in water samples from Southeast Asia: towards an integrated water detection system. Infect. Dis. Poverty 1–12. doi:10.1186/s40249-016-0095-z.
Kwolek-Mirek, M., Zadrag-Tecza, R., 2014. Comparison of methods used for assessing the viability and vitality of yeast cells. FEMS Yeast Res 14, 1068–1079. doi:10.1111/1567-1364.12202.
Lee, G.-C., Nam, S.-H., Chae, J.-C., Lee, C.-H., 2009. Giardia duodenalis: improved detection of viable cysts by reverse transcription-PCR of heat shock-inducible hsp70 gene. Exp. Parasitol. 123, 377–380. doi:10.1016/j.exppara.2009.08.011.
Ligda, P., Claerebout, E., Kostopoulou, D., Zdragas, A., Casaert, S., Robertson, L.J., Sotiraki, S., 2020. Cryptosporidium and Giardia in surface water and drinking water: animal sources and towards the use of a machine-learning approach as a tool for predicting contamination. Environ. Pollut. 264, 114766. doi:10.1016/j.envpol.2020.114766.
Liu, G., Dong, F., Fu, C., Smith, Z.J., 2019. Automated morphometry toolbox for analysis of microscopic model organisms using simple bright-field imaging. Biol. Open 8. doi:10.1242/bio.037788, bio037788.
MacIntyre, H.L., Cullen, J.J., 2016. Classification of phytoplankton cells as live or dead using the vital stains fluorescein diacetate and 5-chloromethylfluorescein diacetate. J. Phycol. 52, 572–589. doi:10.1111/jpy.12415.
Masangkay, F.R., Milanez, G.D., Tsiami, A., Hapan, F.Z., Somsak, V., Kotepui, M., Tangpong, J., Karanis, P., 2020. Waterborne protozoan pathogens in environmental aquatic biofilms: implications for water quality assessment strategies. Environ. Pollut. 259, 113903. doi:10.1016/j.envpol.2019.113903.
McGuigan, K.G., Mendez-Hermida, F., Castro-Hermida, J.A., Ares-Mazas, E., Kehoe, S.C., Boyle, M., Sichel, C., Fernandez-Ibanez, P., Meyer, B.P., Ramalingham, S., Meyer, E.A., 2006. Batch solar disinfection inactivates oocysts of Cryptosporid-

ium parvum and cysts of Giardia muris in drinking water. J. Appl. Microbiol. 101, 453–463. doi:10.1111/j.1365-2672.2006.02935.x.
Medeiros, R.C., Sammarro Silva, K.J., Daniel, L.A., 2020. Wastewater treatment performance in microbiological removal and (oo)cyst viability assessed comparatively to fluorescence decay. Environ. Technol. 0, 1–21. doi:10.1080/09593330.2020.1811396.
Deng, M.Y., Cliver, D.O., 1992. Degradation of Giardia lamblia cysts in mixed human and swine wastes. Appl. Environ. Microbiol. 58, 2368–2374. doi:10.1128/aem.58.8.2368-2374.1992.
Monis, P.T., Caccio, S.M., Thompson, R.C.A., 2009. Variation in Giardia: towards a taxonomic revision of the genus. Trends Parasitol 25, 93–100. doi:10.1016/j.pt.2008.11.006.
Morita, S., Namikoshi, A., Hirata, T., Oguma, K., Katayama, H., Ohgaki, S., Motoyama, N., Fujiwara, M., 2002. Efficacy of UV irradiation in inactivating Cryptosporidium parvum Oocysts. Appl. Environ. Microbiol. 68, 5387–5393. doi:10.1128/AEM.68.11.5387-5393.2002.
Mtapuri-Zinyowera, S., Midzi, N., Muchaneta-Kubara, C.E., Simbini, T., Mduluza, T., 2009. Impact of solar radiation in disinfecting drinking water contaminated with Giardia duodenalis and Entamoeba histolytica/dispar at a point-of-use water treatment. J. Appl. Microbiol. 106, 847–852. doi:10.1111/j.1365-2672.2008.04054.x.
Netuschil, L., Auschill, T.M., Sculean, A., Arweiler, N.B., 2014. Confusion over live/dead stainings for the detection of vital microorganisms in oral biofilms - which stain is suitable? BMC Oral. Health 14, 1–12. doi:10.1186/1472-6831-14-2.
Ongerth, J.E., 2013. The concentration of Cryptosporidium and Giardia in water – the role and importance of recovery efficiency. Water Res 47, 2479–2488. doi:10.1016/j.watres.2013.02.015.
Onichandran, S., Kumar, T., Salibay, C.C., Dungca, J.Z., Tabo, H.A.L., Tabo, N., Tan, T., Lim, Y.A.L., Sawangjaroen, N., Phiriyasamith, S., Andiappan, H., Ithoi, I., 2014. In: Waterborne parasites: a Current Status from the Philippines, 7, pp. 1–8. doi:10.1186/1756-3305-7-244.
Ortega-Pierres, M.G., Jex, A.R., Ansell, B.R.E., Svärd, S.G., 2018. Recent advances in the genomic and molecular biology of Giardia. Acta Trop 184, 67–72. doi:10.1016/j.actatropica.2017.09.004.
Paziewska-Harris, A., Schoone, G., Schallig, H.D.F.H., 2016. An easy 'one tube' method to estimate viability of Cryptosporidium oocysts using real-time qPCR. Parasitol. Res. 115, 2873–2877. doi:10.1007/s00436-016-5044-z.
Pecková, R., Stuart, P.D., Sak, B., Květoňová, D., Kváč, M., Foitová, I., 2016. Statistical comparison of excystation methods in Cryptosporidium parvum oocysts. Vet. Parasitol. 230, 1–5. doi:10.1016/j.vetpar.2016.10.007.
Quilez, J., Sanchez-Acedo, C., Avendaño, C., Del Cacho, E., Lopez-Bernad, F., 2005. Efficacy of two peroxygen-based disinfectants for inactivation of Cryptosporidium parvum oocysts. Appl. Environ. Microbiol. 71, 2479–2483. doi:10.1128/AEM.71.5.2479-2483.2005.
Ramo, A., Del Cacho, E., Sánchez-Acedo, C., Quílez, J., 2017a. Occurrence and genetic diversity of Cryptosporidium and Giardia in urban wastewater treatment plants in north-eastern Spain. Sci. Total Environ. 598, 628–638. doi:10.1016/j.scitotenv.2017.04.097.
Ramo, A., Del Cacho, E., Sánchez-Acedo, C., Quílez, J., 2017b. Occurrence of Cryptosporidium and Giardia in raw and finished drinking water in north-eastern Spain. Sci. Total Environ. 580, 1007–1013. doi:10.1016/j.scitotenv.2016.12.055.
Razzolini, M.T.P., Breternitz, B.S., Kuchkarian, B., Bastos, V.K., 2020. Cryptosporidium and Giardia in urban wastewater: a challenge to overcome. Environ. Pollut. 257, 113545. doi:10.1016/j.envpol.2019.113545.
Robertson, L.J., Campbell, A.T., Smith, H.V., 1992. Survival of Cryptosporidium parvum oocysts under various environmental pressures. Appl. Environ. Microbiol. 58, 3494–3500.
Robertson, L.J., Campbell, T., Smith, H.V., 1993. In vitro excystation of Cryptosporidium parvum. Parasitology 106, 13–19. doi:10.1017/S003118200007476X.
Robertson, L.J., Casaert, S., Valdez-Nava, Y., Ehsan, M.A., Claerebout, E., 2014. Drying of Cryptosporidium oocysts and Giardia cysts to slides abrogates use of vital dyes for viability staining. J. Microbiol. Methods 96, 68–69. doi:10.1016/j.mimet.2013.11.001.
Rochelle, P.A., Upton, S.J., Montelone, B.A., Woods, K., 2005. The response of Cryptosporidium parvum to UV light. Trends Parasitol 21, 81–87. doi:10.1016/j.pt.2004.11.009.
Rousseau, A., La Carbona, S., Dumètre, A., Robertson, L.J., Gargala, G., Escotte-Binet, S., Favennec, L., Villena, I., Gérard, C., Aubert, D., 2018. Assessing viability and infectivity of foodborne and waterborne stages (cysts/oocysts) of Giardia duodenalis, Cryptosporidium spp., and toxoplasma gondii: a review of methods. Parasite 25, 14. doi:10.1051/parasite/2018009.
Ryan, U., Paparini, A., Monis, P., Hijjawi, N., 2016. It's official – Cryptosporidium is a gregarine: what are the implications for the water industry? Water Res. 105, 305–313. doi:10.1016/j.watres.2016.09.013.
Sammarro Silva, K.J., Sabogal-Paz, L.P., 2020a. Giardia spp. cysts and Cryptosporidium spp. oocysts in drinking water treatment residues: comparison of recovery methods for quantity assessment. Environ. Technol. 41, 1–10. doi:10.1080/09593330.2020.1723712.
Sammarro Silva, K.J., Sabogal-Paz, L.P., 2020b. Analytical challenges and perspectives of assessing viability of Giardia muris cysts and Cryptosporidium parvum oocysts by live/dead simultaneous staining. Environ. Technol. 40, 1–18. doi:10.1080/09593330.2020.1775712.
Santos, S.R.Dos, Branco, N., Franco, R.M.B., Paterniani, J.E.S., Katsumata, M., Barlow, P.W., De Mello Gallep, C., 2015. Fluorescence decay of dyed protozoa: differences between stressed and non-stressed cysts. Luminescence 30, 1139–1147. doi:10.1002/bio.2872.
Sena-Lopes, Â., Remião, M.H., Alves, M.S.D., Fonseca, B., da, R., Seixas, F.K., Collares, T., Borsuk, S., 2020. Cell viability analysis of Toxocara cati larvae with live/dead® viability/cytotoxicity kit. Exp. Parasitol. 212, 107871. doi:10.1016/j.exppara.2020.107871.
Shin, G.-A., Linden, K.G., Arrowood, M.J., Sobsey, M.D., 2001. Low-pressure UV inactivation and DNA repair potential of Cryptosporidium parvum oocysts. Appl. Environ. Microbiol. 67, 3029–3032. doi:10.1128/AEM.67.7.3029-3032.2001.
Silva, H.G., Sabogal-Paz, L.P., 2020. Filter backwash water and floated residue containing pathogenic protozoa: detection method and treatment alternatives. Water. Air. Soil Pollut. 231. doi:10.1007/s11270-020-04515-z.
Smith, H.V., Nichols, R.A.B., Grimason, A.M., 2005. Cryptosporidium excystation and invasion: getting to the guts of the matter. Trends Parasitol. 21, 133–142. doi:10.1016/j.pt.2005.01.007.
Smith, H.V., Paget, T., 2007. Giardia, in: Foodborne Diseases. Humana Press, Totowa, NJ, pp. 303–336. doi:10.1007/978-1-59745-501-5_11.
Strober, W., 2015. Trypan Blue Exclusion Test of Cell Viability. Current Protocols in Immunology. John Wiley & Sons, Inc., Hoboken, NJ, USA p. A3.B.1-A3.B.3. doi:10.1002/0471142735.ima03bs111.
Sun, Z., Blatchley, E.R., 2017. Tetraselmis as a challenge organism for validation of ballast water UV systems. Water Res. 121, 311–319. doi:10.1016/j.watres.2017.05.052.
Thompson, R.C.A., Ash, A., 2016. Molecular epidemiology of Giardia and Cryptosporidium infections. Infect. Genet. Evol. 40, 315–323. doi:10.1016/j.meegid.2015.09.028.
USEPA, US Environmental Protection Agency, 2014. Method 1693: Cryptosporidium and Giardia in Disinfected Wastewater By Concentration / IMS / IFA. EPA 821-R-14-013.
USEPA, US Environmental Protection Agency, 2012. Method 1623.1: Cryptosporidium and Giardia in Water by Filtration/IMS/FA EPA/816/R-12/001.
Vande Burgt, N.H., Auer, A., Zintl, A., 2018. Comparison of in vitro viability methods for Cryptosporidium oocysts. Exp. Parasitol. 187, 30–36. doi:10.1016/j.exppara.2018.03.002.
Weir, S.C., Pokorny, N.J., Carreno, R.A., Trevors, J.T., Lee, H., 2002. Efficacy of common laboratory disinfectants on the infectivity of Cryptosporidium parvum oocysts in cell culture. Appl. Environ. Microbiol. 68, 2576–2579. doi:10.1128/AEM.68.5.2576-2579.2002.
Widmer, G., Clancy, T., Ward, H.D., Miller, D., Batzer, G.M., Pearson, C.B., Bukhari, Z., 2002. Structural and biochemical alterations in Giardia lamblia cysts exposed to ozone. J. Parasitol. 88, 1100–1106 10.1645/0022-3395(2002)088[1100:SABAIG]2.0.CO;2.
Wilke, G., Ravindran, S., Funkhouser-Jones, L., Barks, J., Wang, Q., VanDussen, K.L., Stappenbeck, T.S., Kuhlenschmidt, T.B., Kuhlenschmidt, M.S., Sibley, L.D., 2018. Monoclonal antibodies to intracellular stages of Cryptosporidium parvum define life cycle progression in vitro. mSphere 3, 1–14. doi:10.1128/msphere.00124-18.
Wu, M., Bridle, H., Bradley, M., 2012. Targeting Cryptosporidium parvum capture. Water Res 46, 1715–1722. doi:10.1016/j.watres.2011.12.041.
Yaoyu, F., Xiao, L., 2011. Zoonotic potential and molecular epidemiology of Giardia species and giardiasis. Clin. Microbiol. Rev. 24, 110–140. doi:10.1128/CMR.00033-10.
Zawawy, E., El-Said, D., Ali, S., Fathy, F., 2010. Disinfection efficacy of sodium dichloroisocyanurate (NADCC) against common food-borne intestinal protozoa. J. Egypt. Soc. Parasitol. 40, 165–185.