A comparison of two novel pulsed power electrotechnologies for the inactivation of *Cryptosporidium* spp. and other problematical microorganisms in drinking water

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Abstract—The waterborne parasite *Cryptosporidium* represents a significant threat to public health due to its resistance to conventional chlorination. This novel multidisciplinary study investigated the development of pulsed ultraviolet light (PUV) and pulsed-plasma gas-discharge (PPGD) for the novel inactivation of Cryptosporidium oocysts and other problematical microorganisms in water. This constitutes the first study to report on the use of these pulsed power electrotechnologies (PPET) for decontamination of the recalcitrant Cryptosporidium parasite in water

Keywords: Water treatment; pulsed ultraviolet light; pulsed plasma gas discharge; *Cryptosporidium.*

I. INTRODUCTION

Cryptosporidium is a genus of obligate enteric protozoan parasites with species that infect fish, amphibians, reptiles, birds and mammals. *Cryptosporidium* infection was mainly observed in ruminant animals until 1976, when two cases of human cryptosporidiosis were reported [1, 2]. With the increased occurrence rates of Acquired Immunodeficiency Syndrome (AIDS) in subsequent years, cryptosporidiosis became recognised as a causative agent of diarrhoea in immunocompromised individuals. A number of major outbreaks of cryptosporidiosis in the past two decades [3-6] have highlighted the gravity of and dangers associated with *Cryptosporidium* contamination of drinking water.

The relative ineffectiveness of conventional disinfectants such as free chlorine and monochloramine for the inactivation of encysted parasites [7] has led to evaluation of alternative disinfectants for drinking and wastewater treatment [8]. In recent years, novel pulsed power electrotechnologies (PPETs) have been introduced and are being considered as possible alternatives to current methods for inactivating microorganisms in water. PPETs are characterised by the concentration of energy, both in time and space, to pulses of high intensity. The pulsation of energy augments power efficiency and enables new applications that are not possible with conventional continuous flow of energy. By accumulating energy over relatively long periods of time and by dissipating this energy in intense ultrashort pulses (85-100 nanoseconds), the energy remains constant but the peak power increases by several orders of magnitude. During each pulse, very high levels of peak power are generated (10-20 MW), and treatment is achieved using the required number of pulses.

It is proposed that a major breakthrough in the decontamination and sterilisation of drinking water and wastewater can be achieved through the development of novel low-temperature technologies, such as pulsed UV light and pulsed plasma gas-discharge systems.

II. METHODS

A. Pulsed ultraviolet light system

The pulsed-UV system utilized was the PUV-01 (SAMTECH Ltd., Glasgow), and consists of two main components; a treatment chamber and a driver circuit (Fig. 1). The driver unit consists of trigger and discharge outputs, frequency control, trigger control and the discharge voltage control. The trigger cable connects the trigger output of the

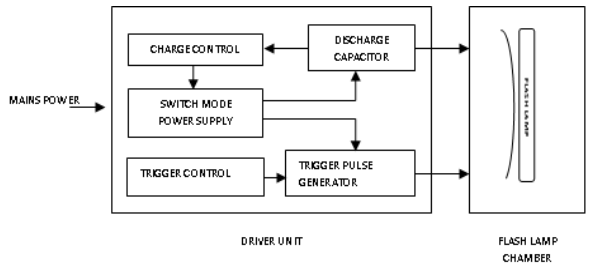

Figure 1: Schematic Diagram of PL-01 system. (Source: Author constructed, adapted from SAMTECH PL-01 User Manual)

driver unit with the trigger electrode of the flashlamp, while the discharge cable connects the discharge output of the driver unit with the lamp anode and cathode. The treatment chamber consists of a polyvinyl-chloride housing containing a xenon light source and a circular treatment table. The treatment table is designed to accommodate a standard Petri dish of 92mm diameter, ensuring stability for samples and aiding in reproducibility. The light source within the chamber is a Heraeus Noblelight XAP (type NL4006) series, consisting of a clear quartz tube filled with xenon to a pressure of 450 torr (59 kPa). The chamber houses a secondary trigger supply that provides the lamp with 25kV pulse to trigger the flashlamp. The light produced by the lamp includes broad spectrum wavelengths form UV to near-infrared. The UV dose can be adjusted by increasing or decreasing the frequency of the pulsing and/or the charging voltage. The pulsed-UV system has the potential of being orders of magnitude more efficient in terms of energy delivery, treatment time, depth of UV penetration, and disinfection efficacy compared to using conventional light sources. The solid state pulsed power source utilizes power compression technology to transfer stored electrical energy to the xenon flashlamp in a short duration but with high peak power.

To prepare the test samples, microbial test strains were streaked to purity from porous beads taken from Microbank vials (Cruinn Diagnostic, Ireland), and an isolated colony was then transferred to 100 ml of an appropriate liquid growth medium. Test microorganisms as well as their respective culture requirements and conditions are summarized in Table 1. Inoculated broths were cultivated with shaking at 125 oscillations per minute at the required temperature

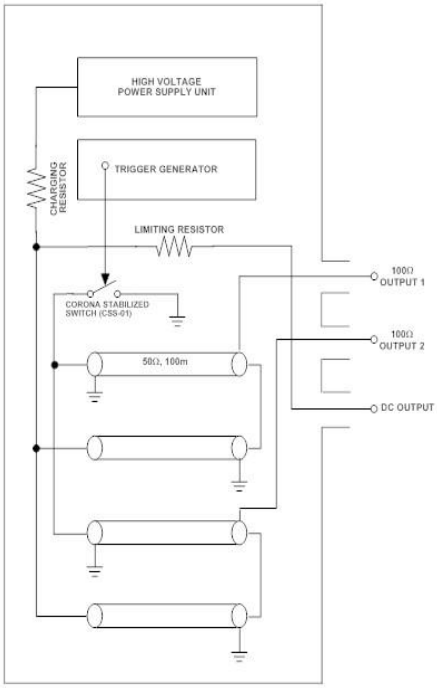

Figure 2: Schematic diagram of internal layout of pulse generator**.** (Source: Author constructed, adapted from SAMTECH Pulsed Plasma Gas Discharge User Manual)

until each test organism reached late exponential phase. The optical densities of test samples were then spectrophotometrically (Model UV-mini 1240 instrument, Shimadzu Corp., Kyoto, Japan) adjusted at 600 nm to 0.8 units (ca. 10^9 CFU/ml) using 0.1 M phosphate buffered saline (PBS) [pH 7.2] (confirmed via aerobic plate count). OD_{600nm} adjusted samples were re-suspended in 20 ml sterile 0.1 M PBS, which was aseptically transferred to 92 mm Petri dishes and subjected to pulsed light treatments. All studies were carried out at a distance of 8 cm from the light source. The number of pulses of light used ranged from 0 (untreated control) to 70 pulses using a lamp discharge energy of 12.8 J that was shown previously to inactivate test yeast populations by ca. 7 log CFU/ml over this treatment regime [9]. Measurement of UV fluence, or dose, (J/cm^2) at each applied pulse was determined using chemical actinometry as described by Rahn *et al*. [10], as the non-continuous emitted spectrum did not facilitate use of a calibrated radiometer.

Table 1: Test micro-organisms used in experiments and their respective culture requirements

Organism	Strain	Media		Incubation Temp.
		Broth	Agar	
Escherichia coli	ATCC 25922	NB[a]	NA[b]	37°C
Pseudomonas aeruginosa	ATCC 27853	NB	NA	37°C
Staphylococcus aureus	ATCC 25923	NB	NA	37°C
Candida albicans	NUHG 8054	MEB[c]	MEA[d]	35°C

[a]Nutrient Broth [b]Nutrient Agar [c]Malt Extract Broth [d]Malt Extract Agar

B. *Pulsed plasma gas discharge system*

The high voltage pulsed power generator used to create the biocidal PPGD is a prototype designed and developed by SAMTECH Ltd (Glasgow, UK). The main components are a high voltage power supply, a set of charging resistors, a trigger generator linked to a corona stabilised switch (CSS) and a pulse generator. The pulse generator (Fig. 2) is composed of a set of pulse forming network transmission lines of a Blumlein configuration. This PPGD system produces multiple shortlived biocidal properties in the treatment chamber that includes ozone, acoustic shock waves, ultraviolet light and pulsed electric fields [11].

The PPGD test liquid was prepared by transferring 1 ml of a ca. 10^9cfu/ml microbial suspension, prepared as above, into 99 ml of an ice cold dH_2O/PBS (96%/4% v/v) solution. The PPGD treatment chamber was attuned to house a treatment volume of 100 ml with the top of the needle electrodes arranged exactly 5 mm above the test liquid. The chamber was connected to the discharge gas and the required gas flow rate was adjusted. The SF_6 pressure within the CSS was matched to the desired charging voltage. The pulse repetition rate was set on the trigger unit panel and the pulse generator was initiated. Periodically, samples were aseptically withdrawn to determine the number of surviving bacterial cells/oocysts.

C. *Temperature*

Before and during the PUV and PPGD treatment respectively, temperature of the treatment fluid was periodically monitored to ascertain the potential mechanisms that would underlie microbial inactivation. Temperature was monitored by using an Eutech® CyberScan pH 510 pH/mV meter with automated temperature control adjustment (Thermo Fischer Scientific, UK).

D. *Cryptosporidium oocysts*

C. parvum oocysts (Iowa isolate derived from a bovine calf) were purchased from Waterborne Inc, USA. Oocysts were stored in sterile PBS (0.01 M phosphate buffer, containing 0.0027 M KCL and 0.137 MNaCl at a pH of 7.4) with 100 U of penicillin/ml, 100 µg of streptomycin/ml and 100 µg of gentamicin/ml and stored at 4 °C until they were used for UV treatment studies.

E. *In vitro cell culture infectivity assay*

Prior to PUV treatment, *Cryptosporidium* oocysts were diluted to a final volume of 20 ml (ca. 1×10^5 oocysts/ml) in sterile 0.1M PBS. The oocyst suspension was placed on the treatment table (distance from UV light source: 8 cm) inside the treatment chamber of the PUV system and was flashed with pulses of broad-spectrum light. Prior to PPGD treatment, *C. parvum* oocysts were diluted to a final volume of 100 ml in dH_2O/PBS (96%/4% v/v) solution (ca. 1×10^5 oocysts/ml), aseptically transferred to the treatment chamber and pulsed as above. Periodically, samples were aseptically withdrawn to determine the number of surviving oocysts. Following either type of PPET treatment, the oocyst suspension was transferred to a sterile centrifuge tube (Sarstedt) wrapped in aluminium foil to prevent photoreactivation and centrifuged at 3000 rpm for 15 minutes. The oocysts were resuspended in 1 ml sterile acidified HBSS, pH 2.7 and incubated for 1 hour at 37°C. The oocysts were again centrifuged at 3000 rpm for 15 minutes at room temperature and the pellet resuspended in 1 % w/v bile salts (Sigma) (pH 7.0) in mammalian cell culture media for 15 minutes at 37 °C. After two washing steps with sterile PBS, the oocysts were finally resuspended in 0.5 ml of cell culture media and a haemocytometer count performed.

Caco-2 cells (human colon adenocarcinoma [12], ATCC HTB-37: American Type Culture Collection, Rockville, Md, USA) were grown in T75 cell culture flasks (Sarstedt) containing cell culture media Dulbecco modified Eagle's medium/Ham's F-12 medium, supplemented with 20% (v/v) foetal bovine serum, 10 g/L L-glutamine, 1% (v/v) non-essential amino acids, supplemented antibiotics (penicillin G, 100,000 U/L, streptomycin, 0.5 g/L

and amphotericin B, 0.5 g/L); adjusted to pH 7.4. Cells were maintained in a humidified incubator at 37 °C in an atmosphere containing 5% (v/v) CO_2 for ca. 24 h until 80 to 90% confluent monolayers had formed. Cell monolayers were then detached with 0.25% (v/v) trypsin-EDTA and subsequently seeded into each of 8 well chambered slides (Lab Tec II, Nunc) at a concentration ca. 1×10^5 cells per well, as determined by haemocytometer counts. The chamber slides were incubated as above for 12 hours. Cell culture media was removed from the cell monolayers in the wells without disturbing the and the cells were washed once with sterile PBS, then 0.5 ml of media containing the treated oocysts was then added per well. Untreated oocysts were also stimulated (via HBSS pH 2.7 and 1% w/v bile salts) to infect the cell monolayer as described above and provided a control. One sample of oocysts was heat treated at 70°C for 30 minutes and this preparation was used as a negative control (as such treatment was shown previous to inactivate the parasite by Rochelle [13]) allowing for a comparative cell line infectivity study using dead oocysts. The chamber slides were incubated at 37°C and 5% CO_2 for 24 hours, after which the media was removed from the monolayer which was subsequently, washed using sterile PBS and fresh media added for further 24 hour incubation.

F. Staining of oocyst infected cell monolayer

After 48 hour incubation, the chamber slides were removed from the incubator and the cell media was removed from the monolayer by pipetting of the media using sterile pipettes (Sarstedt). Each individual well containing a separate monolayer was fixed by flooding the well with 100% methanol (Sigma) which was subsequently left to stand for 10 minutes at room temperature. After fixation the methanol was removed and 75 µl of the Sporo-Glo™ A600FLR-20X (Waterborne Inc, USA) immunofluorescent stain was added to each monolayer which was subsequently incubated at 37°C for 45 minutes. Sporo-Glo™ stain is used for evaluating the viability of *C. parvum* in sterile slide chambers with mammalian monolayers and uses the principle of direct immunofluorescence. The reagent consists of a fluorescein labelled rat monoclonal antibody which binds to all life cycle stages of the parasite *in vitro*; the specimens will appear bright green when viewed under fluorescent microscopy. After staining the monolayers were washed in sterile PBS. Once removed from the PBS, cell monolayers were counterstained with C101 counter-stain (Waterborne Inc, USA) containing Evans blue (which stains the cell monolayer red and allows for a background when viewing the fluorescent green oocysts) for 1 minute followed by a 1 minute washing step using sterile PBS. All slides were examined under fluorescence microscopy (Leithz Diaplan fluorescence microscope) at excitation wavelength of 460 to 500 nm and an emission wavelength of 510 to 560 nm for Sporo-Glo™ and an excitation wavelength of 550 nm and emission wavelength of 610nm for the counterstain C101. All chamber wells containing separate monolayers were examined and noted as positive or negative for sites of parasitic infection. The number of fluorescent foci (representing parasitic life cycle stages) was assessed and rates of infection for untreated controls and treated samples were determined. A monolayer was recorded positive for both sporozoite invasion and clustering of foci of infection indicating completing of the oocysts life cycle, as per [14]. Images of *C. parvum* life cycle stages *in vitro* were taken with the use of a camera (Hamamatsu Colour Chilled 3cco Camera) attached to the fluorescence microscope.

III. RESULTS AND DISCUSSION

Species utilized in this study were chosen to represent a broad range of potentially pathogenic organisms to include Gram negative bacteria, Gram positive bacteria, and eukaryotic yeast species (as described in Table 1). All are common contaminants of potable water [15-18]. These test microorganisms differ genetically, morphologically and metabolically (including adaptive stress responses) and it is these combined properties that make some more sensitive to the external environment than others.

A. PPET inactivation of microorganisms in suspension

Results presented in this study show that all test strains which were artificially seeded in liquid suspensions were reduced by the lethal action of pulsed UV light. The microbial response to pulsed UV exposure can be modelled as a single stage exponential decay. The first-order disinfection

model of Chick [19] and Watson [20] was used to investigate microbial population survival. The inactivation of micro-organisms is

$$\log_{10}[N_t/N_0] = -kt \qquad (1)$$

where N_t is microbial concentration after exposure time **t**, N_0 is concentration prior to treatment and **k** is the rate constant. The inactivation rate (k) is the average $\log_{10}$ reduction in cell viability as a function of exposure time. The rate constant defines the sensitivity of a microorganism to pulsed UV intensity and is unique to each microbial species. The inactivation rate (k) achieved at 12.8J per pulse for each microbial species is shown in Table 4. The inactivation rate (k) was found to decrease in the following order: *E. coli* > *S. aureus* > *P. aeruginosa* > *C. albicans.*

The short pulse width and high doses of the pulsed UV source can provide some practical advantages over PPGD disinfection in those situations where rapid disinfection is required. The variation in wavelengths supplied by PUV light may also have a broader range of damaging effects on bacterial and protozoan organisms e.g. there is a possibility that the repair mechanisms may be affected by irradiation at different wavelengths. While variation in PUV inactivation performances can be attributed to multiple inter-related factors including differences in UV irradiation method and species, the duration of UV exposure at any applied dose appears paramount for reducing or eliminating infectivity. Therefore, it is quite apparent that effectively reducing or eliminating the threat of low numbers of *C. parvum* in contaminated water at full treatment plant scale level will demand using an extensive number of continuously operating PUV lamps (run in parallel) in addition to

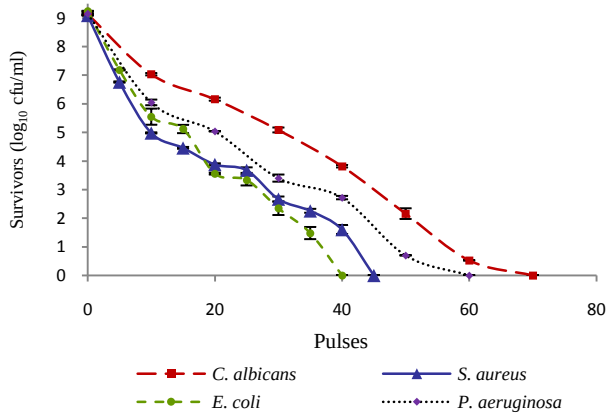

Figure 3: Pulsed UV inactivation of microorganisms; applied voltage 800 V; 12.8J per pulse; 1 pulse per second; distance from light source 8 cm; fluence rate = 2.4021 mW/cm^2

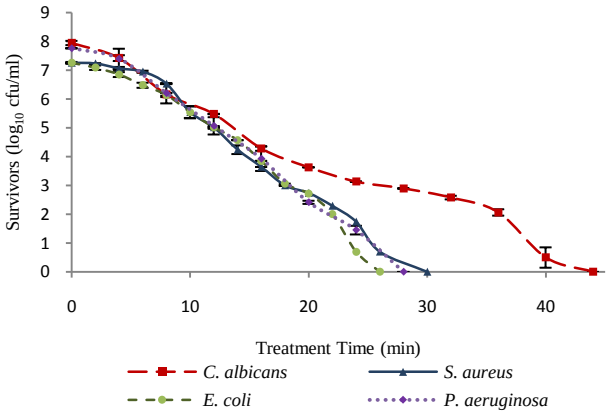

Figure 4: Pulsed plasma gas discharge inactivation of microorganisms; PFN charging voltage 16 kV; 10 pulses per second; sparged gas O_2 2.5 L/min.

possibly reducing the flow rate of water to fall within the minimum disinfection efficacy threshold of the PUV system.

The PPGD inactivation experiments show that an effective reduction in the surviving microbial cell density was evident for all microorganisms tested. It was observed, that after an initial shoulder effect, the microbial densities decreased in an almost linear fashion with increasing treatment time. The shoulder in the curve generally represents the period in which the microorganisms receive a sub-lethal dose and little or no inactivation occurs [21]. The inactivation rate (k) achieved at 18 kV PFN charging voltage, 2.5 L/min O_2 and 10 pulses per second for each microbial species is shown in Table 3. The inactivation rate (k), according to equation (1) was found to decrease in the following order: *P. aeruginosa* > *S. aureus* > *E. coli* > *C. albicans.*

There are several mechanisms which have been reported to contribute to the deleterious effects of gas-discharge plasmas on microorganisms: These include the interaction with chemically active substances, including ozone, hydrogen peroxide, hydroxyl and superoxide free radicals, DNA damage caused by UV radiation generated by the discharge itself, and nonspecific effects resulting from the formation of pressure shockwaves [22]. The application of an external electric field to microorganisms in suspension can cause irreversible electrical breakdown of the cell membrane and lead eventually to the death of the

Table 2: Summary of inactivation rate constant *k* values for microorganisms using pulsed UV light; 800 V, 12.8 J per pulse (+/- S.E).

Microorganism	k value	R^2
E. coli	0.1807 ± 0.14	0.97
S. aureus	0.1389 ± 0.06	0.96
P. aeruginosa	0.1363 ± 0.07	0.99
C. albicans	0.1209 ± 0.07	0.99

Table 3: Summary of inactivation rate constant *k* values for microorganisms using pulsed plasma gas discharge; 16 kV, 10 pps, 2.5 L/min O_2 (+/- S.E).

Microorganism	k value	R^2
E. coli	0.2629 ± 0.071	0.99
S. aureus	0.2835 ± 0.065	0.99
P. aeruginosa	0.3075 ± 0.075	0.99
C. albicans	0.1510 ± 0.048	0.96

cell [23]. This mechanism is known as electroporation.

B. Temperature

Due to the broad-spectrum nature of the PUV system, including both visible and infrared light, as well as ultra-violet light, the potential for sample heating was investigated. There was an increase of 0.4°C after 140 pulses at a discharge voltage of 800 V (data not shown). This infers a temperature increase of 0.0029°C per pulse in a 20 mL sample, and it is reasonable to assume that this rate would decrease when pulses are applied to larger volumes of water. These results indicate such a minute temperature increase that is probable that microbial cell death is not due to the thermal effects related to PUV treatment, but due to some other effect.

Within as little as 20 minutes of PPGD treatment at 16 kV, the temperature of the test liquid rose by 24.1°C (data not shown). The rise in temperature had to be considered in terms of microbial inactivation, to ensure that the cause of death in microorganisms was a direct result of plasma discharge application rather than simply due to excess heat. Thus, it was of the utmost importance to monitor the temperature rise within the chamber during the treatment. While a small amount temperature increase was expected due to Joule heating (a process whereby when a pulse is applied to the test chamber, current flows and some of the energy from the pulse is always converted to heat), the results of the temperature studies are of concern in terms of this system's application in microbial decontamination. A temperature increase above 40°C may contribute to microbial death during PPGD treatments; therefore it becomes difficult to elucidate the specific physicochemical methods by which the PPGD causes microbial death and the rates at which they do so.

C. PPET inactivation of C. parvum as determined by in vitro infectivity

Untreated and treated oocysts were stimulated to infect a cell monolayer (in quadruplicate) and stained with immunofluorescent stains to verify the presence or absence of parasitic life cycle stages (foci of infection). To determine if parasitic infection occurred pre and post UV exposure monolayers were examined and scored as positive or negative for the presence or absence of parasitic life cycle stages respectively. Each monolayer was also monitored for the presence of clusters of parasitic life cycle stages. The clustering effect is indicative of numerous life cycle stages infecting the same area and is due to the formation of type I and type II meronts and thin walled oocysts *in vitro* which continue to autoinfect or re-infect the monolayer.

Infection of the monolayer with *C. parvum* produced a vast number of infective life cycle stages, of various sizes. Due to the numerous foci of infection present in each monolayer following exposure to *C. parvum* oocysts it was not possible to quantify the numbers of foci present. As was also reported by Schets *et al.,* [14] non-synchronous excystation and infection, and secondary sites of infection resulted in the production of large clusters of various reproductive stages. It was therefore impossible to relate the number of foci present in a well to the number of infectious oocysts present in the sample originally applied to the monolayer. Therefore, assessment the level of inactivation was carried out by an adaptation of the methods of Schets *et al*., [14] and Garvey *et al*., [24]. The presence of clusters of infection was also recorded per monolayer. This indicates that the oocysts are not only capable of initial cellular invasion of the cell cultures but also of proliferating to further life cycle stages (macrogametes and or thin walled oocysts) and therefore, resulting in auto-infection or re-infection of the cell monolayer.

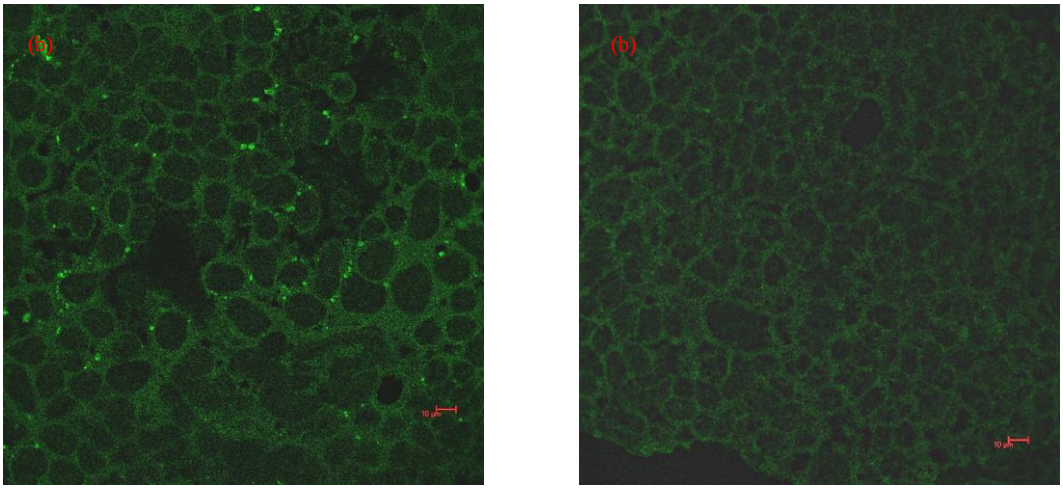

Figure 5: (a) Caco-2 cell monolayer infected with untreated *Cryptosporidium* oocysts. (b) Caco-2 cell monolayer infected with PUV-treated *Cryptosporidium* oocysts (90 pulses at 12.8J per pulse). Monolayers were stained with Sporoglo® fluorescein-labelled polyclonal rat IgG antibody and Evans Blue counterstain

Table 4: Infectivity of PUV treated *C. parvum* in Caco-2 cell monolayers.

Pulses	UV Fluence ($\mu J/cm^2$)	Infected Monolayers	Level of infectivity	Presence of clusters
0	0.000	4	High	+
15	1.261	4	High	+
30	2.522	4	High	+
45	3.783	3	Medium	+
60	5.044	3	Medium	-
75	6.306	2	Low	-
90	7.567	1	Low	-
120	10.089	0	None	-

Table 5: Infectivity of PPGD treated *C. parvum* in Caco-2 cell monolayers. Charging voltage was 16kV. Pulse frequency was 10 pps. Gas utilized was O_2 at 2.5 L/min.

Treatment Time (min)	Infected Monolayers	Level of infectivity	Presence of clusters
0	4	High	+
8	4	High	+
16	3	Medium	+
24	2	Medium	-
32	1	Low	-

A clear pattern was observed in this study where more rapid reductions in populations of *C. parvum* oocysts occurred with increasing levels of pulsing, using either PPET system. Similar samples of oocysts that were subjected to heating at 70 °C for 30 min did not infect Caco-2 cells (negative control). Complete inactivation (5 log reduction) was achieved within 120 pulses using PUV. Figure 5 depicts images obtained via fluorescent microscopy (control and PUV treated oocysts respectively). However, after 32 minutes of PPGD treatment, equating to 19,200 pulses, a low level *Cryptosporidium* viability remained. This suggests that PUV is more efficient in the inactivation of *Cryptosporidium* oocysts than PPGD in terms of time and energy. Also, considering the very low numbers of ingested oocysts required to cause cryptosporidiosis, nothing less than complete inactivation would be acceptable in terms of drinking water treatment. In a noted human infectivity study, the infectivity and morbidity resulting from oral ingestion of *Cryptosporidium* oocysts in adult human volunteers were evaluated. The lowest single dose tested in this study (30 oocysts) initiated infection in 20% of those individuals exposed [25]. In more recent studies, it was demonstrated that as few as 10 oocysts can cause infection in otherwise healthy adults [26].

This project has generated critical data on the efficacy of PUV and PPGD-treated water that will facilitate the advancement of water research and will impact strongly on public health by safeguarding water quality and supplies.

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