Efficacy of measuring cellular ATP levels to determine the inactivation of pulsed UV treated *Cryptosporidium parvum* oocysts suspended in water

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**ABSTRACT**

This constitutes the first study to report on the use of a novel approach to determine inactivation in PUV-irradiated *Cryptosporidium parvum* oocysts suspended in water based on the measurement of cellular adenosine triphosphate (ATP) concentration. This study also compares the efficiency of a novel ATP assay to that of using the combined *in vitro* HCT-8 cell culture – quantitative polymerase chain reaction (qPCR) method for determining the inactivation in the waterborne pathogen *C. parvum* after exposure to pulsed UV (PUV) treatments. Findings were compared with using the combined cell culture-qPCR approach for determining oocyst viability in similarly treated samples. PUV effectively killed *C. parvum* with a 5.4 log$_{10}$ loss in oocyst viability after exposure to a UV dose of 8.5 μJ/cm$^2$ as determined by the *in vitro* cell culture – qPCR assay. The ATP assay was shown to be significantly less effective in measuring loss of oocyst viability in similarly PUV-irradiated samples for all combination of treatment regimes studied. Measurement of cellular ATP is not suitable as an indicator of the disinfection efficiency of PUV-irradiated *C. parvum* oocysts. The levels of ATP present post PUV-irradiated samples suggests that significant cellular activity remained in treated oocysts that are unable to invade human HCT-8 cells. However, further studies are merited to investigate factors that might aid repair post PUV treatments in this water-borne human parasite. Use of this ATP assay offers an interesting insight into loss of infectivity in PUV-treated *C. parvum*. This rapid assay does not appear suitable for investigating or optimizing treatment efficiency under varying operational settings as it detects PUV-treated oocysts at levels significantly higher compared with using the *in vitro* cell culture-qPCR infectivity assay. Overestimation of survivors by the ATP assay may suggest that a sub-population of *C. parvum* oocysts may exist in a viable but non-infectious state or may require a period of resuscitation to facilitate photo-repair (if possible) that may lead to regained ability to infective human hosts.

**Key words** | ATP assay, combined cell culture-qPCR, *Cryptosporidium*, pulsed UV light, viability

**INTRODUCTION**

*Cryptosporidium* is a coccidian parasite that is now a well-recognized cause of diarrhoea in immunocompetent and immunocompromised humans (*Fayer et al. 2007*). Also *Cryptosporidium* species have been known to cause serious disease conditions on numerous occasions resulting from the consumption of contaminated drinking water (*MacKenzie et al. 1994*). It has been documented that *Cryptosporidium parvum* represents a threat to public health in the water industry due to the very low infection dose of oocysts and its resistance to standard drinking water treatment methods (*Hijawi 2003; Lee et al. 2008*). Studies of water sources in the UK and US have found that oocysts
commonly occurred in all types of surface water with densities as low as 2.5 oocysts/L (Gray 2005). Also it is known that low levels of *C. parvum* are required for host infectivity (DuPont *et al.* 1995). If the water industry is to make accurate assessments of the risk to public health posed by waterborne *Cryptosporidium* it must be able to determine not just their presence and viability but also whether the oocysts are capable of causing infection. Viability and infectivity assays will also provide the water industry with a tool to measure the efficiency of disinfection protocols (Rochelle *et al.* 1997). Therefore, it is essential to develop such methods that will contribute to the elimination of this parasite from drinking water supplies.

Use of conventional low pressure (LP) UV light has been found to be effective at inactivating a range of problematic organisms including *Cryptosporidium* oocysts but suffers from operational limitations (Rochelle *et al.* 2005). Other novel approaches of UV-irradiation have been developed in recent times such as medium-pressure (MP) and pulsed UV (PUV; Hijnem *et al.* 2006). Optimization of these emerging approaches has been affected by the lack of availability of simple rapid methods to detect and enumerate viability of *C. parvum* post UV treatments, which is currently limited to complex *in vitro* cell culture combined with and real-time quantitative polymerase chain reaction (RT-qPCR) (Keegan *et al.* 2003; Garvey *et al.* 2010). Measurement of oocyst death and associated loss of parasitic infectivity in host tissues is important when investigating the efficiency of traditional and new processes for killing *Cryptosporidium*. Therefore, establishing whether or not treated oocysts in water are viable post-treatments governs decontamination efficiency. Previous studies have reported on the use of range of methods including immunofluorescence microscopy and colorimetric endpoints for detecting *Cryptosporidium* (Fontaine & Guilot 2003) however, these sophisticated non-viability approaches are time-consuming and require use of highly specialist equipment and technique. An animal model using infectivity in mice was developed and considered the gold standard for assessing oocyst viability post decontamination but this approach is also cumbersome and raises significant ethical concerns (Karanis & Aldeyarbi 2011). Therefore, this study explored ways in which to repeatedly and accurately determine oocyst viability following exposure to PUV (Figure 1). One alternative or complimentary candidate method is the measurement of cellular adenosine triphosphate (ATP) levels as all living cells contain ATP, which is the basic unit of energy currency in viable cells. ATP is not present in non-viable cells, as it is degraded after death. ATP has been used as an indicator of viability of microorganisms including *Cryptosporidium* (Holm-Hansen & Booth 1966; Patterson *et al.* 1970; Weddle & Jenkins 1971; King *et al.* 2005). ATP measurement is a likely candidate method for rapidly determining the viability or activity of this parasite pre- and post-disinfection particularly as oocyst excystation requires the generation and use of ATP. Indeed, King *et al.* (2005) reported previously that the quantification of oocyst ATP levels can provide a simple and rapid method to estimate oocyst inactivation post heat treatment. Also, Somiya *et al.* (2000) concluded that the ATP assay is superior to *in vitro* excystation and 4,6-diamidino-2-phenylindole/propidium iodide (DAPI/PI) permeability assays because of its rapid and simple procedure. Based on the aforementioned, the aim of this study was to assess the potential of an ATP assay to detect the concentration of ATP in treated and untreated *C. parvum* oocysts, in order to determine loss in viability following exposure to PUV irradiation. Use of the ATP measurement may alleviate the strong dependency of complex *in vitro* cell culture and or mice infectivity studies for determining PUV disinfection efficiency. Disinfection levels as determined via ATP measurement pre- and post-UV exposure were also compared with the combined *in vitro* HCT-8 cell culture qPCR assay which was shown previously to correlate with the gold standard mouse infectivity model (Garvey *et al.* 2010).

**MATERIALS AND METHODS**

**Preparation viability staining of *C. parvum* 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. Use of a combined surrogate dye staining method comprising PI and a fluorescein-labelled mouse-derived monoclonal antibody A400FLR-1X Crypt-a-Glo™ (having corresponding epiteope oocyst cell...
wall; Waterborne Inc., New Orleans, USA) was used to confirm the viability of oocysts. The excystation rate was determined for each batch of oocysts by microscopic observation following sequential incubation at 37 °C in acidified Hanks’ balanced salt solution (HBSS) for 1 h and in 0.8% trypsin–0.75% sodium taurocholate for 1 h, followed by incubation at room temperature for 30 min as described elsewhere (Rochelle et al. 2002). For negative infection studies, oocysts were inactivated by heating at 70 °C for 30 min. All experiments were carried out using oocysts with greater than 80% viability, as determined by in vitro excystation as per Korich et al. (2000).

**Enumeration and viability of *C. parvum* oocysts**

Standard counts were determined for all oocyst stocks. Viable oocysts were enumerated by serial dilution in phosphate buffered saline (PBS) containing the aforementioned antibiotics using both fluorescence microscopy (confocal microscopy, Leica DM 600 CS, Germany), and real-time qPCR post infection in HCT-8 cell line as per method of Garvey et al. (2010). Oocyst suspensions at different population sizes were initially centrifuged at 3,000 rpm for 15 min at 4 °C, then re-suspended in acidified HBSS pH 2.7 for 1 h at 37 °C before filtration onto polycarbonate black 0.8 μm pore-size membrane filters (Fischer Scientific) using a filter system (Millipore) at a vacuum pressure of 200 mbar (1 bar = 105 Pa). Ten microlitres of PI (Sigma), prepared by dissolving 1 mg of PI in 1 ml of 0.1 M sterile PBS, and one drop (circa 45 μl) of A400FLR-1X Crypta-Glo™ were then applied to oocysts on the membrane and incubated for 2 h at room temperature in the dark. The membranes were mounted on glass microscope slides with 4 μl mounting medium (glycerol [non-photoreactive], 2 ml; 100 mg of DABCO (1,4-diazabicyclo[2,2,2] octane) per ml of double distilled water, 2.4 ml; 0.1 ml Tris buffer, 4.8 ml; formalin 0.5 ml; and 5 MNaCl, 0.5 ml) and sealed with coverslips and clear nail varnish. Non-viable oocysts stained bright red due to uptake of PI when viewed at an excitation wavelength of 460–500 nm and an emission spectrum of 510–560 nm. Whereas all viable and non-viable oocysts stained apple-green when stained with A400 FLR-1X at an excitation wavelength of 460–500 nm and an emission wavelength of 510–560 nm. Entire membranes were scanned and all oocysts suspensions were counted by fluorescence microscopy. All counts were determined in triplicate.

**Pulsing of *C. parvum* samples with UV rich light**

A bench-top pulsed power source (PUV-1, Samtech Ltd, Glasgow) was used to power a LP (60 kPa) xenon-filled flashlamp (Heraeus Noblelight XAP type NL4006 series constructed from a clear UV transparent quartz tube), that produced a high-intensity diverging beam of polychromatic pulsed light, was used in this study as per Garvey et al. (2010). The pulsed light has a broadband emission spectrum extending from the UV to the infrared region with a rich UV content and its intensity also depends on the level of the
voltage applied (Figure 1). The light source has an automatic frequency control function that allows it to operate at 1 pulse per second (pps) that was used throughout this study. Light exposure was homogenous as the xenon lamp measuring 9 x 0.75 cm was longer than the 8.5 cm standard diameter. Petri dishes used in the tests which were placed directly below the lamp, which ensured that full coverage of the agar surface occurred and eliminated possible shading effects. For standard treatments (unless otherwise noted), the light source was mounted at 8 cm above the treatment area, as this distance was shown previously to be optimal for inactivation of C. parvum (Garvey et al. 2010). In this study, standard treatments involved suspending predetermined numbers of C. parvum in 10 ml of PBS that were transferred to Petri dishes that was then subjected to lamp discharge energies of 16.2 J, and 20 J at 8 cm distance from the light source up to and including a fluence of 25.92 and 34 μJ/cm² at a rate of 1 per second respectively. Heating of the oocyst suspensions was measured using a thermocouple and by thermal imaging (IRI 4010, InfraRed Integrated Systems Ltd, Northampton, UK) as per modified method of Nugent & Higginbotham (2007). There was no discernable increase in water temperature during each treatment. All studies were run in triplicate.

ATP assay

The ATP Biomass Kit HS (BioThema, Luminescent Assays, Handen, Sweden) was used to detect and quantify ATP in PUV treated and untreated controls following a method of King et al. (2005) with modifications. This assay is based on the principle of luminescence utilizing the firefly luciferin-luciferase which is known to be highly specific (Somiya et al. 2000). A standard curve of ATP concentration vs. oocysts concentration was prepared by serial dilution of circa. 10⁶ oocysts/ml through to 10¹ oocysts/ml in sterile PBS. Final 1 ml volumes of oocyst dilutions were centrifuged at 3,000 rpm for 15 min in a microcentrifuge (Microcentrifuge, MSE Sanyo, Micro Centaur), subsequently the supernatant was removed by careful pipetting and the pellet resuspended in 1 ml of acidified HBSS (pH 2.7). The samples were vortexed for 10 s to disperse the oocyst pellet and a cell count performed using a haemocytometer to ensure that serial dilutions produced the correct quantity of oocysts per ml. All samples were incubated at 37 °C for 1 h to allow excystation to occur. After incubation, samples were again centrifuged at 3,000 rpm for 15 min, the supernatant was removed and oocysts resuspended in 100 μl of sterile MilliQ water. One hundred microlitres of extractant B/S (ATP Biomass Kit) was added to each sample and vortexed for 10 s, subsequently samples were sonicated (5510 Branson Sonicator) for varying times (30, 60, and 90 s) to determine optimal conditions for ATP extraction (Somiya et al. 2000). Following sonication, the ATP assay was conducted as per the manufacturer’s instructions with some minor alterations. Specifically, 40 μl of the oocyst extractant solution was removed from the sample container and added to one well of the 96 well plate in triplicate, 160 μl of reconstituted ATP Reagent HS was added to each well containing oocyst samples. The light emission produced by the addition of the ATP reagent to each well containing C. parvum ATP was immediately measured using a luminometer (Wallac Victor™, 1420 Multilabel counter). Once the reading was obtained (Iₛmp₁) the light emission was again read after 5 s (Iₛmp₂) and 10 μl of the 100 nmol/L ATP standard (supplied with kit) was added to each well and the light emission measured (Iₛmp₁std) after mixing. In triplicate a blank was also run containing only the medium in which the oocysts were suspended, i.e. MilliQ water, the purpose of this was to ensure no external ATP was present in the medium. All ATP studies were conducted in the dark using black 96 well plates as luciferin is rapidly degraded by bright light.

Once the assay was complete the amount of ATP (pmol) per sample was calculated using the equation:

\[
\text{ATP}_{\text{sample}} = \frac{I_{\text{smp}1}}{I_{\text{smp}1\text{std}} - I_{\text{smp}2}}
\]

Pulsed UV inactivation studies were then conducted by treating 1 ml samples containing circa. 10⁶ oocysts/ml with a range of pulses at 16.2 and 20 J respectively (corresponding UV doses shown in Tables 1 and 2). After treatment oocyst samples were assayed for ATP content as per the method described above. The amount of ATP (pmol) present after PUV inactivation was used to determine the reduction in oocyst viability post UV exposure by linear regression analysis utilizing the standard curve obtained by measuring ATP (pmol) concentration per oocyst concentration. All studies were conducted in triplicate.
Table 1  Log_{10} reduction in C. parvum viability following exposure to pulses of UV light at varying at UV dose at 16.2 J as detected by a cell culture RT-PCR assay and ATP assay. Results are a mean of 3 replicates (± S.D.)

<table>
<thead>
<tr>
<th>No. of pulses</th>
<th>UV dose μJ/cm²</th>
<th>Log_{10} reduction in viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>1.08</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>30</td>
<td>3.24</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>40</td>
<td>4.32</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>60</td>
<td>6.48</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>90</td>
<td>9.72</td>
<td>5.4 ± 0.1</td>
</tr>
<tr>
<td>120</td>
<td>12.9</td>
<td>-a</td>
</tr>
<tr>
<td>150</td>
<td>16.2</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>180</td>
<td>19.44</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>210</td>
<td>22.6</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>240</td>
<td>25.92</td>
<td>3 ± 0.1</td>
</tr>
</tbody>
</table>

*No detectable target gene present.

Table 2  Log_{10} reduction in C. parvum viability following exposure to pulses of UV light at varying at UV dose at 20.0 J as detected by a cell culture RT-PCR assay and ATP assay. Results are a mean of 3 replicates (± S.D.)

<table>
<thead>
<tr>
<th>No. of pulses</th>
<th>UV dose μJ/cm²</th>
<th>Log_{10} reduction in viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>1.42</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>30</td>
<td>4.26</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>40</td>
<td>5.68</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>60</td>
<td>8.52</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>90</td>
<td>12.78</td>
<td>-a</td>
</tr>
<tr>
<td>120</td>
<td>17.04</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>150</td>
<td>21.3</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>180</td>
<td>25.5</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>210</td>
<td>29.8</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>240</td>
<td>34</td>
<td>3.8 ± 0.1</td>
</tr>
</tbody>
</table>

*No detectable target gene present.

In vitro cell culture infectivity

Cell culture infectivity was confirmed by immunofluorescent staining of treated HCT-8 cell monolayers following exposure to viable oocysts. Monolayers of the human ileocecal adenocarcinoma cell line HCT-8 (ATCC CCL-244: American Type Culture Collection, Rockville, MD) were grown with regular subculturing in RPMI 1640 growth media with L-glutamine and supplemented antibiotics (penicillin G, 100,000 U/L, streptomycin, 0.5 g/L and amphotericin B, 0.5 g/L), sodium bicarbonate, 2 g/L, and 10% foetal calf serum adjusted to pH7.4. HCT-8 cells were grown in T75 cm⁻² cell culture flasks in a humidified incubator at 37 °C in an atmosphere containing 5% (vol/vol) CO₂ for circa 24h until 80–90% confluent monolayers had formed. Cell monolayers were then detached with 0.25% (vol/vol) trypsin–EDTA and subsequently seeded into each of eight well chambered slides (Lab Tec II, Nunc) at a concentration circa 1 x 10⁵ cells per well. After UV or heat treatments, the oocysts were stimulated by re-suspension in acidified HBSS (pH 2.7) and then in 1.0% (wt/vol) bile salts (pH 7) for 1 h at 37 °C. After two washing steps with sterile PBS, oocysts were re-suspended in cell culture media and thereafter 350 μl aliquots were then added to each well. Untreated oocysts were also stimulated to infect the cell monolayer as described above and provided a positive control. Duplicate sample of oocysts was heat treated at 70 °C for 30 min and this preparation was used as a negative control as per Rochelle et al. (2002). Samples were incubated for up to 48 h at 37 °C in 5% (vol/vol) CO₂ atmosphere, to determine optimal conditions for cell infectivity. At set times each individual well containing a separate monolayer was fixed by flooding with 100% (vol/vol) methanol (Sigma) which was subsequently left to stand for 10 min at room temperature. After removal of methanol, 75 μL of the fluorescein stain Sporo Glo™A600FLR-20X (Waterborne Inc., UK) was added to each well for 45 min (at 37 °C), which detects different life cycle stages of Cryptosporidium in vitro. The inoculated HCT-8 cell monolayers were then counterstained for 1 min with C101 containing Evans blue dye (Waterborne Inc., USA). All slides were examined under fluorescence microscopy (Leitz Diaplan fluorescence microscope) at an excitation wavelength of 460–500 nm and an emission wavelength of 510–560 nm for SporoGlo™ and an excitation wavelength of 550 nm and emissions wavelength of 610 nm for the counterstain C101. All wells containing separate monolayers was examined and noted as positive or negative for sites of parasitic...
infection or foci of infection. Images of *C. parvum* life cycle stages were captured using a camera (Hamamatsu Colour Chilled 3cco camera) mounted on the aforementioned fluorescence microscope. All studies were performed in triplicate.

**Combined cell culture-quantitative PCR (CC qPCR) assay for enumerating viable *C. parvum* post treatments**

Real-time, TaqMan-quantitative PCR (qPCR) was performed using primers (TIB MOLBIOL, Berlin, Germany) specific for the 18S region of *Cryptosporidium* following the method of Keegan *et al.* (2005) with some slight modifications. Real-time PCR reactions are characterized by an increase in fluorescence emission due to probe degradation by DNA polymerase in each elongation step during PCR cycling. The higher the starting copy number of the nucleic acid target, the earlier the fluorescence will reach the predetermined threshold and the smaller will be Ct. The threshold cycle (CT) is the fractional PCR cycle number at which a significant increase in target signal fluorescence above the baseline is first detected for a sample. Quantification of test samples is performed by determining the Ct value and the use of a standard curve to deduce the starting copy number. The sequence of the TaqMan probe was based on the conserved eukaryotic probe of Amman *et al.* (1990) with the following sequence: 5'-(6-FAM) ACC AGA CTT GCC CTC C (TAMRA). An aliquot (4 μl) of the Lightcycler TaqMan® Master kit (Roche Diagnostics, West Sussex, UK) comprising Taq DNA polymerase, reaction buffer, MgCl₂ and dNTP was used in each reaction. Cycling parameters were initial denaturation for 10 min at 95 °C followed by 50 cycles of denaturation for 10 s at 95 °C, annealing for 40 s at 40 °C, extension for 1 s at 70 °C and cooling for 30 s at 40 °C on a LightCycler® device (Model 1.5, Roche Diagnostics, West Sussex, UK). The large number of cycles was used to ensure detection of low levels of infection. On completion of each RT-PCR run amplification curves were analysed by LightCycler® software (version 3.5, Roche) and a standard curve of oocyst DNA concentration determined. When required, PCR amplicons were visualized by UV illumination following electrophoresis in 1% agarose gels containing ethidium bromide. DNA standards were prepared from fresh oocysts ranging in concentration from 10 to 10⁷ oocysts/ml by dilution in PBS following standard viable count determinations. Aliquots of oocysts at different densities were then stimulated to infect the HCT-8 cell line that were seeded into 24 well plates at a concentration of circa 1×10⁴ cells/ml at 90% confluency. The latter cell line stimulation occurred by resuspension and separate incubations for 1 h in acidified HBSS and in bile salts as described earlier. One millilitre aliquots of each concentration range of excysted oocysts were re-suspended in

![Figure 2](https://iwaponline.com/ws/article-pdf/13/2/202/415987/202.pdf)

**Figure 2** | Standard curve for *C. parvum* oocyst number vs. ATP concentration (pmol).
RPMI cell culture growth media and added to one well of the 24 well plate. Following 48 h incubation at 37 °C in a humidified atmosphere of 5% (vol/vol) CO2, the cell culture media with non-adherent or internalized C. parvum was removed by aspiration and discarded. Mammalian cells were then washed with sterile PBS and trypsinized using 200 μl of 0.25% (vol/vol) trypsin/EDTA (Sigma) and left for 15 min at 37 °C until complete detachment of the monolayer had occurred. Cells were then centrifuged at 1,000 rpm for 10 min and re-suspended in 200 μl sterile PBS, thereafter the mammalian cells and C. parvum sporozoite cell membranes were lysed using PCR template preparation kit (Roche Diagnostics, West Sussex, UK) in order to produce DNA (template) and standard curve (Figure 3). The aforementioned procedure was then repeated to determine infectivity of oocysts subjected to varying UV parameters or heating at 70 °C for 30 min (negative control). Log inactivation of oocysts (L) is defined by $L = \log_{10} \left( \frac{N_0}{N_d} \right)$, where $N_0$ is the initial concentration of oocysts and $N_d$ is the concentration of viable infectious oocysts post disinfection treatments as detected by combined cell culture-qPCR assay as per method of Lee et al. (2008).

**RESULTS AND DISCUSSION**

**Determination of rate of PUV inactivation of C. parvum using a combined cell culture real-time PCR in vitro assay**

A standard curve of C. parvum oocyst number and corresponding DNA amplification was generated by using a combined cell culture (CC) - qRT-PCR procedure by inoculating HCT-8 monolayers with different concentrations of C. parvum oocysts (Figure 3). Based on the number of oocysts added to each PCR reaction for the DNA standards, relative quantification of infectious oocysts was performed for estimating log inactivation of oocysts during treatment processes. The uninfected HCT-8 cell extract failed to produce a detectable signal in the PCR, indicating no amplification of the host cell DNA with the 18S primers (data not shown). Following UV treatments, the Ct values obtained for PUV-treated oocysts were higher compared with the untreated control suggesting that there was a decrease in the number of viable oocysts present after UV treatment (Table 1). Using the 16.2 J per pulse setting (Table 1), a 1.2 log10 reduction in viability or infectivity was obtained post PUV-irradiation with a UV dose of 3.24 μJ/cm² while a maximal 5.4 log10 reduction in oocysts infectivity was achieved using the higher UV dose of 9.72 μJ/cm² (Figure 4). At the higher discharge voltage of 1,000 V (or 20 J per pulse) (Table 2), a 1.2 log10 reduction in oocyst infectivity was achieved using a significantly lower UV dose (1.42 μJ/cm²) and a maximal 5.6 log10 loss in oocyst infectivity occurred after exposure to a UV dose of 8.52 μJ/cm². At UV doses 8.52 μJ/cm², no C. parvum DNA was detected in the host cell monolayer via the combined CC-qPCR compared with untreated controls (Figure 4) suggesting that once complete oocyst inactivation was achieved no cell infectivity occurred resulting in an absence of target DNA for amplification. Mofidi et al. (2001) also used HCT-8 cell cultures and real-time PCR assay to evaluate the efficiency of UV light as a disinfectant against C. parvum and demonstrated an average level of inactivation of 94% (1.2 log10) using a similar polychromatic UV system that emitted a UV dose of 4 mJ/cm. Therefore, pulsed UV light successfully inactivated C. parvum oocysts as determined via the cell

![Figure 3](https://iwaponline.com/ws/article-pdf/13/2/202/415887/202.pdf)
culture qPCR assay. For this PCR assay, the limit of detection was found to be 10 oocysts per monolayer. The log inactivation rate for each type of UV treatment was determined using the equation previously described (Garvey et al. 2010). The results demonstrate that the assay was highly reproducible, evident by the linear standard curve obtained for serial dilution of the stock culture (Figure 3). The HCT-8 cell line failed to produce a detectable signal in the PCR, indicating no amplification of host cell DNA with the 18S primers. Also heat inactivated oocysts included as the experimental negative controls were not detected indicating successful removal of oocysts that had not undergone excystation. To date most published data on inactivating C. parvum is using LP and MP UV light sources, Craik et al. (2001), Keegan et al. (2003) and Mofidi et al. (2001) reported that a UV doses of 6, 5.8 and 10 mJ/cm² respectively provided an average 2 log₁₀ loss of viability of C. parvum (Lee et al. 2008). The results of this study indicate that with a pulsed UV approach, similar levels of inactivation occur with a greatly reduced applied UV dose, e.g. 1.42 μJ/cm² gave a 1.2 log₁₀ loss in viability following a 10 s exposure at 20 J per pulse.

Previous studies conducted comparing in vitro cell culture alone or combined cell culture-qPCR to that of measuring infectivity in live severe combined immunodeficiency (SCID) mice found good correlation between PUV inactivation as determined by both these methods (Rochelle et al. 2002; Garvey et al. 2010). Such studies further support the findings of this research and confirm the disinfection potential of PUV for C. parvum and suggest that the optimal method to determine infectivity post treatment is the use of a cell culture based qPCR method. Studies conducted by Fontaine and Guillot (2002) to assess the sensitivity of RT-PCR, for viability of C. parvum oocysts also found a similar detection limit to that of this study. Also studies conducted by Keegan et al. (2003) observed a detection limit of 10 oocysts per inoculated monolayer using a cell culture qPCR assay, confirming that this assay allows for effective quantification of infection by direct comparison of disinfected and untreated oocysts in cell culture. Therefore, the findings of this study demonstrate that cell culture combined with qPCR allows determination of the inactivation of C. parvum oocysts using UV light. These findings are consistent with the findings of Lee et al. (2008) and demonstrate that qPCR combined with in vitro cell culture is a valuable tool for evaluating disinfection systems for drinking water treatment. While this complex approach is widely accepted as an effective and reliable method for determining inactivation of C. parvum oocysts, its usage is restricted to specialist-equipped laboratories operated by highly-trained technicians. Therefore, it is important to assess alternative methods for oocyst inactivation studies.

**ATP measurement for viability assessment**

Adenosine-5’-triphosphate (ATP) is a multifunctional nucleotide that acts as a coenzyme and an energy source...
which is consumed by many enzymes in a multitude of cellular processes. A linear relationship was observed between ATP (pmol) extracted by the method of excystation and sonication and oocyst number over the range of 10,000,000–10 oocysts (Figure 2). These findings correspond to that of Somiya et al. (2000) where the ATP sonication procedure used had a linear relationship to that of the DAPI/PI permeability assay for oocyst viability. Tables 1 and 2 show the relationship between UV dose for PUV-treated samples and associated reduction inactivation rates of treated oocysts. As evident from Table 2 and Figure 5, a maximal 3.8 log inactivation rate was achieved using 34 μJ/cm² at 20 J (Figure 5) whereas a maximal 3 log reduction in similarly prepared samples was achieved using a UV dose of 25.92 μJ/cm² at 16.2 J per pulse (Table 1). The general trend emerged where the ATP assay significantly overestimated survivors compared with using the combined in vitro cell culture qPCR infectivity assay. In addition, it took circa seven times more UV dose to register a 3 log reduction in oocyst cell infectivity as detected using the ATP assay compared with using the more sensitive cell culture-qPCR method.

Studies conducted previously by Somiya et al. (2000) found that ATP assays also overestimated oocyst viability compared with SCID mouse infectivity models following disinfection with ozone. As with the findings of King et al. (2005), the level of sensitivity for the detection of C. parvum via measurement of ATP was found to be circa 500 oocysts. The lower level of sensitivity for this test meant that the inactivation data were grossly underestimated. This suggests that the level of ATP degradation within UV treated oocyst does not occur rapidly enough to be used as a viability endpoint post UV treatment. The findings of King et al. (2005) suggest that measurement of ATP levels can allow for the assessment of oocyst inactivation following heat treatment of C. parvum. These studies reported a decline in oocyst infectivity of HCT-8 cells with a corresponding decline in oocysts ATP levels. King et al. (2005) concluded that the quantification of oocyst ATP levels could provide a simple and rapid method for determining oocyst inactivation rates and suggests that ATP is utilized by the parasite following changes in environmental temperature and as a result is not available as an energy source for excystation. However, the mechanisms of heat inactivation are dissimilar to that of UV light where the formation of DNA adducts by the latter prevents parasitic replication. Therefore, the findings of this study suggest that the measurement of ATP may not be a sufficient method to determine oocyst inactivation following PUV-irradiation as determined by comparative cell culture qPCR studies. The inaccuracy of ATP measurement post UV exposure may be related to the rate of degradation of ATP in UV-treated organisms. At present there are limited data available on the cellular activity of UV-treated oocysts, and the fate of such essential cell components as ATP.

Figure 5 | Reduction in C. parvum oocyst viability 16.2 and 20 J by pulsed UV light at a distance of 8 cm from the light source (+/− S.D.) as detected by measurement of ATP.
Due to constant exposure to solar UV, microorganisms have developed mechanisms to repair genetic damage caused by the absorption of photons of UV energy. Conventional UV treatment methods largely affect DNA by means that are reversible under certain conditions, due to exposure to UV light organisms such as Cryptosporidium have the ability to conduct DNA repair (Oguma et al. 2001). Indeed after treatment with UV light, Cryptosporidium will have initiated those repair mechanism. The role of ATP within cells is such that it is essential for all cellular mechanisms. Therefore, the measurement of ATP levels within PUV treated oocysts indicates that damaged oocysts may still remain active but incapable of performing cellular functions such as invading human cells. Studies by Oei & Ziegler (2000) reported that most situations requiring highly efficient DNA repair are accompanied by a dramatic decrease of the cellular ATP concentration. This corresponds with the findings of this study and suggests that the reduction in ATP levels following high doses of UV (Tables 1 and 2) are due to the energy requiring activity within the organism such as photo repair as opposed to actual cell death. Indeed as with the findings of King et al. (2005), this may suggest that at higher PUV treatment levels the ATP is no longer available as an energy source for excystation due to the demand for photo-repair. The findings of Waldstein et al. (1974) concluded that ATP is required in vitro for either the incision step of photo-repair or an enzymatic reaction preceding it in UV treated E. coli. Studies conducted by Farrell et al. (2011) on the effect of PUV on yeast concluded that pulsed irradiation inactivates C. albicans through a multi-hit cellular process that includes conflicting irreversible damage to DNA and destabilizing the functionality and integrity of plasma cell membranes. The decrease in available ATP suggests a secondary method to that of the formation of DNA adducts for the inactivation of Cryptosporidium with UV light. Without sufficient energy reserves at the moment of cellular infectivity sporozoites are unable to initiate infection and/or reproduction within a host. To date there is no data on the effect of high doses of pulsed light on the membrane and internal structures of Cryptosporidium.

The ability to initiate and conduct repair caused to DNA following UV disinfection is an important aspect to consider when sterilizing drinking water. The comparative qPCR assay used for this study show that at lower PUV doses (<9.72 μJ/cm²) no cell infectivity occurred and that Cryptosporidium had lost its ability to be infective. The author believes that the depletion of ATP following high UV doses (34 μJ/cm²) in this study suggest that research is needed to determine the potential of Cryptosporidium to repair any UV-induced genetic damage and to regain infectivity in host cells. These findings have significant implications for PL-technology development, in particular for surface and water decontamination applications.

**CONCLUSIONS**

- Using an ATP assay, it was possible to measure the intracellular ATP content of viable oocysts where a linear relationship between oocyst number and ATP content was determined. However, the findings of this study demonstrated that the measurement of intracellular ATP is not suitable for the determination of PUV disinfection of Cryptosporidium oocysts.
- The level of ATP reduction in PUV-treated oocysts did not correspond to loss of oocyst infectivity as detected by the in vitro cell culture q-PCR assay. Specifically, the ATP assay overestimated oocysts numbers post treatments compared with that determined by the cell culture-qPCR approach.
- The measurement of cellular ATP did indicate that cellular activity was present after treatment and suggests that further studies are required to determine the mechanistic effects of oocyst damage inflicted by PUV irradiation. Cryptosporidium species possess the ability to repair UV induced DNA damage following treatment with conventional low and MP light sources. Consequently this suggests that studies focusing on the DNA repair mechanisms of Cryptosporidium should be conducted following PUV exposure to determine the potential and rate of photorepair and subsequent possibility of regaining infectivity.
- It has been shown that the cell culture approach has equivalency with the gold standard in vivo mice model for measuring loss of oocyst infectivity following UV disinfection.
• This study concludes that ATP measurement is not suitable for assessing oocyst inactivation post PUV-irradiation.

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