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EFFICACY OF USING HARMLESS BACILLUS ENDOSPORES AS NOVEL SURROGATE ORGANISMS TO INDICATE THE INACTIVATION PERFORMANCE OF RECALCITRANT CRYPTOSPORIDIUM PARVUM OOCYSTS SUSPENDED IN WATER USING PULSED UV LIGHT

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Corresponding Author:	mary garvey Athlone Institute of Technology Athlone, Westmeath IRELAND
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Athlone Institute of Technology
Corresponding Author's Secondary Institution:	
First Author:	mary garvey
First Author Secondary Information:	
Order of Authors:	mary garvey Eoghan Clifford Edmond O'Reilly Neil Rowan
Order of Authors Secondary Information:	
Abstract:	<p>The need to use complex in vitro cell culture, expensive equipment and highly-trained technicians that are available only to specialist laboratories has significantly limited studies assessing the potential of pulsed UV light (PUV) to inactivate the waterborne parasite <i>Cryptosporidium parvum</i> in drinking water. This constitutes the first study to report on the use of different non-pathogenic <i>Bacillus</i> endospores as potential surrogate organisms to indicate the PUV inactivation performance of <i>C. parvum</i> oocyst suspended in water. Findings showed that PUV effectively inactivated ca. 5 log₁₀ CFU/ml <i>B. megaterium</i> and <i>B. pumilus</i> endospores suspended in water at a UV dose of 9.72 μJ/cm² that also inactivated statistically similar levels of <i>C. parvum</i> oocysts (P < 0.05) as determined by combined in vitro HCT-8 cell culture and qPCR. Specifically, this study demonstrated that <i>Bacillus megaterium</i> exhibited greater or similar PUV-inactivation kinetic data compared to that of similarly treated <i>C. parvum</i> over the UV dose range 6.4 to 12.9 μJ/cm². Therefore, the former may be used as indicator organism for safely investigating the PUV-inactivation performance of this chlorine-resistant waterborne parasite at waste water treatment plant level. Findings presented will impact positively on future water quality studies and on public health.</p>

GARVEY ET AL. –ENDOSPORES AS SURROGATES FOR C. PARVUM STUDIES

**EFFICACY OF USING HARMLESS BACILLUS ENDOSPORES AS NOVEL
SURROGATE ORGANISMS TO INDICATE THE INACTIVATION PERFORMANCE
OF RECALCITRANT CRYPTOSPORIDIUM PARVUM OOCYSTS SUSPENDED IN
WATER USING PULSED UV LIGHT**

Mary Garvey^{1*}, Eoghan Clifford², Edmond O'Reilly², Neil J Rowan¹

¹Department of Nursing and Health Science, Athlone Institute of Technology, Ireland

²Department of Civil Engineering, National University College Galway, Ireland

*Corresponding author's mailing address:

Department of Nursing and Health Science, Athlone Institute of Technology,

Dublin Road, Athlone, Co. Westmeath, Ireland.

Phone: 00353906442546

Fax: 00353906486135

Email: mgarvey@ait.ie

ABSTRACT:

The need to use complex *in vitro* cell culture, expensive equipment and highly-trained technicians that are available only to specialist laboratories has significantly limited studies assessing the potential of pulsed UV light (PUV) to inactivate the waterborne parasite *Cryptosporidium parvum* in drinking water. This constitutes the first study to report on the use of different non-pathogenic *Bacillus* endospores as potential surrogate organisms to indicate the PUV inactivation performance of *C. parvum* oocyst suspended in water. Findings showed that PUV effectively inactivated ca. 5 log₁₀ CFU/ml *B. megaterium* and *B. pumilus* endospores suspended in water at a UV dose of 9.72 μJ/cm² that also inactivated statistically similar levels of *C. parvum* oocysts ($P < 0.05$) as determined by combined *in vitro* HCT-8 cell culture and *qPCR*. Specifically, this study demonstrated that *Bacillus megaterium* exhibited greater or similar PUV-inactivation kinetic data compared to that of similarly treated *C. parvum* over the UV dose range 6.4 to 12.9 μJ/cm². Therefore, the former may be used as indicator organism for safely investigating the PUV-inactivation performance of this chlorine- resistant waterborne parasite at waste water treatment plant level. Findings presented will impact positively on future water quality studies and on public health.

Protozoan cysts such as *Cryptosporidium* constitute the most commonly identified cause of waterborne diseases in developed countries. *Cryptosporidium* may enter surface waters from urban runoff, agricultural runoff, wastewater discharges, leaking septic systems, and via direct fecal waste from animal and human origin [Sunderland, 2007]. In the well developed countries of America and Western Europe where drinking treatment facilities are designed primarily for bacterial removal the incidence of large scale waterborne bacterial gastroenteritis is now rare. However, over the recent years there has been increasing concern over the emergence of new forms of enteritis caused by protozoan parasites and enteroviruses [Mawdsley, 1995]. The presence of *Cryptosporidium* oocysts has led to many public health outbreaks in both drinking and recreational waters (Schets et al., 2004). Chlorine based compounds have, for many years,

been widely used as the main disinfectants for drinking water systems. However, the recalcitrant nature of the *Cryptosporidium* parasite means that it is resistant to these standard water treatment processes (Me´ndez-Hermida et al., 2007).

Recently, new approaches such as ozonation and pulsed UV light (PUV) have been developed for the sterilization of drinking water. Studies including those carried out by this research group are being conducted on the potential of PUV to inactivate *Cryptosporidium parvum* (Garvey et al., 2010). However, due to the complex nature of the oocyst determination of viability post decontamination has proved challenging with a necessity to apply sophisticated *in vitro* cell culture or *in vivo* mice infection models post treatments [Garvey et al, 2010]. Routine monitoring and detection of (oo)cysts in the aquatic environs has been restricted to specialist laboratories with highly-trained technicians using sophisticated equipment and methodologies [Facile et al. 2000]. Current sampling and analytical methods that are available to detect *Cryptosporidium* have proven both insensitive and inaccurate [Nieminski et al. 2000]. The lack of real-time reliable measurements of recalcitrant pathogens in contaminated water inhibits local authorities in reacting to such remedial needs in terms of drinking and waste-water optimization at treatment plant level. Additionally, oocysts are generally not present in sufficient numbers in water sources to allow direct measurement of *C. parvum* oocyst, which also impacts negatively on the ability to investigate their inactivation performance post PUV treatments. Consequently, there is a requirement to artificially-spike water at pilot WWTP level with this waterborne pathogen unless a surrogate less-fastidious microorganism exhibiting similar PUV inactivation kinetic properties to *C. parvum* can be identified. Thus, research findings from on-going investigations in our research group have highlighted the need to identify and use appropriate surrogate microorganism to serve as a harmless indicator model for *C. parvum* oocysts, particular when investigating scale up parameters from laboratory to pilot waste-water treatment level avoiding concerns of untreated *C. parvum* being released back into municipal water (Irish EPA grant 2011-W-MS-5).

It has been common practice in the past to use indicator organisms such as coliforms to assess the microbiological quality of treated drinking water. In principle, the surrogate microorganism must share similar or preferably an augmented resistance kinetic profile to that the target organisms for treatment methods applied such as PUV. The inactivation kinetics of the non-pathogenic surrogate organism should also be technically easy to measure. Previous studies

have assessed the use of aerobic bacterial spores to monitor the efficiency of ozone and concluded that these organisms serve as both reliable and simple indicators to that of both *Giardia* and *Cryptosporidium* under similar treatment conditions (Facile et al. 2000). *Bacillus subtilis* spores and *Clostridium perfringens* spores have been suggested as possible indicators for *Cryptosporidium*, due to their observed resistance to disinfectants and adverse environmental conditions (Verhille, 2003). This author also reported that the predominant aerobic spore-forming bacteria present in surface and ground water were members of the genus *Bacillus*, whereas the anaerobic endospores from the genus *Clostridium* were found in lower numbers. Indeed, *Bacillus* and *Clostridium* endospores were routinely isolated in treated water, demonstrating their resistance to commonly-used treatment methods [Verhille, 2003]. *C. perfringens* is a known pathogen and therefore not suitable for water disinfection studies at treatment plant level. *Cryptosporidium* are mostly present at low concentrations in raw water, therefore, their monitoring along the drinking water treatment process cannot assess large logarithmic removal efficiencies as needed for disinfection/removal studies [Mazoua, 2005].

Consequently, the aim of this study was to investigate the use and relationship of two harmless aerobic endospore forming *Bacillus* species (namely, *B. pumilus* and *B. megaterium*) as surrogate indicator microorganisms to determine the PUV- inactivation performance of *C. parvum* in artificially-spiked water samples. For comparative PUV- analysis, a pathogenic strain of *B. cereus* was also studied.

MATERIALS AND METHODS

PUV light system

For all studies described a bench-top pulsed power source (PUV-1, Samtech Ltd., Glasgow) was used to power a low pressure (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 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. The light source has an automatic frequency control function that allows it to operate at 1 pulse/s that was used throughout this study. Light exposure was homogenous as the xenon lamp measuring 9 cm×0.75 cm was longer than the 8.5 cm diameter. Petri dishes used in the tests were placed

directly below the lamp, which ensured that full coverage of the plate surface occurred and eliminated possible shading effects. For inactivation studies the light source was mounted at 8 cm above the treatment area, which was set as the minimum threshold distance by the fabricant. In this study, standard treatments involved suspending predetermined numbers of each test species in 20 ml of PBS that were transferred to petri dishes that was then subjected to lamp discharge energies of 16.2 J at 8 cm distance from the light source. The UV dose can be adjusted by increasing or decreasing the frequency of the pulsing and/or the charging voltage.

Preparation and PUV-treatment of vegetative *Bacillus* species

Bacillus megaterium ATCC 14581, *Bacillus pumilus* ATCC 14884 and *Bacillus cereus* ATCC 11778 were sourced from the National Collection of Type Cultures and were selected for this research due to their ability to grow at ambient temperature and for their relative large size that facilitates direct microscopic observations. The former two strains are non-pathogenic, which allows for their use in the study of water disinfection techniques. Strains were grown to single colonies Nutrient agar (Oxoid) at 30°C and their identity confirmed by determining characteristic morphological, physiological and biochemical tests as described previously by Rowan et al. (2001). For PUV studies a single colony of each test strain was separately transferred to 100 ml sterile Nutrient broth followed by incubation at 30°C for 24 hr. Samples were then centrifuged at 3000 rpm for 10 min and the pellet resuspended in 20 ml sterile phosphate buffered saline (0.01 M phosphate buffer, containing 0.0027 M KCL and 0.137 M NaCl at a pH of 7.4) (PBS) to give a working stock with ca. 1×10^8 CFU/ml. Neat and PBS-diluted samples were irradiated at 8 cm from the light source at 16.2J per pulse with 1 pulse delivered per second..

Endospores of *B. cereus*, *B. pumilus* and *B. megaterium* were cultivated as per the method of Rowan et al. (2001) with modifications. Endospores were prepared by growing test strains in Nutrient agar (Oxoid) supplemented with 3 mg/L of manganese sulphate (Sigma) for 4 days at 30°C. Spores were then collected by flooding the agar plate with 10 ml sterile PBS (pH 7), mixing gently with a sterile spatula and then aseptically transferred to sterile 50 mL polypropylene containers (Sarstedt). Samples were then heated to 90°C for 25 min to inactivate any residual vegetative cells present and then washed twice by centrifugation at $1,300 \times g$ for 20 min before resuspending in sterile PBS. The purity of spore suspensions was checked using malachite green spore staining and phase contrast microscopy as described by Rowan et al

(2001). Samples were stored in Microbank™ vials at -80°C. PUV studies were performed as described as described earlier by resuspending endospores at a concentration of ca. 10⁸ CFU/ml in 20 mls sterile PBS (determined spectrophotometrically at 580nm). This allowed for comparative studies to that of *C. parvum* oocysts suspensions in 20 ml volumes. Levels of inactivation were determined by plotting the log₁₀ ratio of survivors against UV dose (μJ/cm²) for each experimental organism.

***Cryptosporidium parvum* oocysts**

C. parvum oocysts (Iowa isolate derived from a bovine calf) were purchased from Waterborne Inc USA. Oocysts were stored in sterile PBS 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. 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 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). Identification of *C. parvum* oocysts was confirmed by PCR targeting of a 620 bp polymorphic region of the β-tubulin gene by previously published procedures (Rochelle et al., 1997) [data not shown]. For negative infection studies, oocysts were inactivated by heating at 70 °C for 30 min. All experiments were carried using oocysts with greater than 80% viability, as determined by *in vitro* excystation as per Garvey et al. (2010) and Korich et al. (2000).

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

Cell culture qPCR was conducted as per described by Garvey *et al.* (2010) using HCT-8 cell monolayer's as host cells for parasitic infectivity followed by real time PCR. Methods for the growth and maintenance of HCT-8 uninfected and infected cells are therefore not described herein. 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 Garvey et al. (2010) and Keegan et al. (2003). 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, England) 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, England). The large number of cycles was used to ensure detection of low levels of infection. On completion of each qPCR run amplification curves were analyzed by LightCycler® software (version 3.5, Roche) and a standard curve of oocyst DNA concentration determined. DNA standards were prepared from fresh oocysts ranging in concentration from 10¹ 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 (Sarstedt) at a concentration of ca. 1×10⁴ cells/ml at 90% confluency as per method of Garvey et al (2010). 1 ml aliquots of each concentration range of excysted oocysts were re-suspended in 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 cell 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 1000 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, England) in order to produce DNA (template) and standard curve (Figure 1). 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) as per method of Garvey *et al*, (2010). Samples were also res-suspended in PBS containing 10% w/v humic acid in order to investigate the influence of organic matter on microbial inactivation as per Garvey et al. (2010). Log inactivation of oocysts (L) is defined by $L = \log_{10}[N_d/N_o]$, where N_o 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).

Statistical analysis

Student's t-tests and ANOVA one-way model (MINITAB software release 16; Mintab Inc., State College, PA) were used to compare the effects of the relationship of independent variables on light treatments. Mean results from three separate experiments were determined. Linear regression analysis was used to determine the rate of inactivation for each test species under the regime of PUV treatments applied.

Results

PUV inactivation of bacterial endospores and vegetative cells

Findings showed a direct relationship between an increase in UV dose and corresponding increase in microbial inactivation in all vegetative forms of *Bacillus* strains tested (Table 1). A general trend was observed where at lower UV doses ($\leq 10.8 \mu\text{J}/\text{cm}^2$) there was a significant difference in the sensitivity of each vegetative test strain to UV exposure with *B. cereus* exhibiting more resistance compared to similarly treated *B. megaterium* and *B. pumilus* ($P < 0.05$) (Table 1). However, at higher UV doses, similar rates of inactivation were obtained for all *Bacillus* strains tested. A UV dose of $10.8 \mu\text{J}/\text{cm}^2$ as required to produce a 6- \log_{10} reduction in CFU/ml for all vegetative strains where the order of sensitivity from most resistance to least was *B. cereus*, *B. pumilus* and *B. megaterium* ($P < 0.05$).

A general trend was observed where *Bacillus* endospores proved more UV resistant than their vegetative state counterparts, whereby an increased UV dose was needed to achieve similar rates of inactivation in similarly PUV-treated vegetative cells (Table 2). For example, a 0.4, 0.54 and 1 \log_{10} reduction in *B. cereus*, *B. megaterium* and *B. pumilus* at $1.08 \mu\text{J}/\text{cm}^2$ respectively, which was ca. 2 log lower compared to similarly PUV-treated vegetative cells. PUV-irradiance at $10.8 \mu\text{J}/\text{cm}^2$ produced a 5.1, 5.3 and 6.7 \log_{10} reduction in endospore numbers for *B. cereus*, *B. megaterium* and *B. pumilus* respectively. This general trend also persisted as with enhanced pulsing the order of increased resistance to PUV was *B. pumilus*, *B. megaterium* and *B. cereus* ($P < 0.05$). At higher UV exposures (such as $10.8 \mu\text{J}/\text{cm}^2$) a 5.1, 5.3 and 6.7 \log_{10} inactivation was obtained for *B. cereus*, *B. megaterium* and *B. pumilus* spores respectively. However, at doses exceeding $12.9 \mu\text{J}/\text{cm}^2$ there was no significant difference between PUV-inactivation performance for test *B. megaterium* and *B. cereus* endospores. While *B. pumilus* endospores

appeared the most UV sensitive for all regimes tested, *B. megaterium* proved the most UV resistant at the uppermost UV doses tested ($19.4 \mu\text{J}/\text{cm}^2$) (Table 2).

Pulsed UV inactivation of *Cryptosporidium parvum* as determined by use of a combined in vitro cell culture-qPCR assay

For relative comparison of samples, a standard curve was generated by inoculating HCT-8 monolayers with different concentrations of *C. parvum* oocysts and performing linear regression analysis of sample mean CT values plotted against numbers of inoculated oocysts (Figure 1). A general trend was observed where an increase in UV exposure produced an increase in oocyst inactivation (Table 2). A UV dose of $9.72 \mu\text{J}/\text{cm}^2$ resulted in a ca. 5 log reductions in oocyst viability. At UV doses exceeding $12.96 \mu\text{J}/\text{cm}^2$ no parasitic DNA was detected in HCT-8 mammalian cells indicating that a a. 6 \log_{10} reduction in oocysts occurred (Table 2), thus showing that PUV successfully and repeatedly inactivated *C. parvum* oocysts. At low UV doses ($\leq 4.32 \mu\text{J}/\text{cm}^2$), *C. parvum* exhibited greater resistance to similarly-treated *Bacillus* endospores. However, *C. parvum* oocysts exhibited lower or similar resistance to *B. megaterium* endospores over the UV dose range 6.48 to $12.9 \mu\text{J}/\text{cm}^2$ (medium to high PUV-irradiance). The inclusion of 10% w/v humic acid (H.A.) did not affect the PUV-inactivation performance of all test organisms compared to control samples devoid of H.A. (Fig. 2). *C. parvum* oocysts were significantly more resistant to PUV than *B. pumilus* at all applied treatment doses.

Discussion

The findings of this study showed a general trend where endospores of each *Bacillus* strain tested proved more UV resistance than their corresponding vegetative form. Also there was a significant difference in individual strain sensitivity to UV treatment for both culture forms at lower treatment doses of $< 10.8 \mu\text{J}/\text{cm}^2$. Endospores of *B. pumilus* consistently showed lower levels of resistance to pulsed UV compared to *B. megaterium* and *B. cereus* tested. In the vegetative state the order of increasing sensitivity to pulsed UV was as follows *B. cereus*, *B. pumilus* with *B. megaterium*. This may be related in part to the larger size of *B. megaterium* cells relative to the other *Bacillus* species tested (Rowan et al., 2001). However, for endospore inactivation studies, the order of increased sensitivity was observed to be *B. megaterium*, *B. cereus* with *B. pumilus*.

This study also corroborates the findings of Sharifi-Yazdi and Darghahi (2006) who reported that *B. megaterium* endospores proved more resistant to UV irradiation compared to similarly treated *B. cereus* endospores. These researchers achieved a 2 log₁₀ CFU/ml reduction of *B. megaterium* with 50 pulses (7.1 μJ/cm²) and a 5 log₁₀ following 25 pulses (3.2 μJ/cm²) for *B. cereus* at 20J per pulse. While, the mechanisms underpinning destruction of spores differs to that of similarly UV-treated vegetative cells, DNA remains the principal target with the formation of a lethal adjunct of a thymine dimer in spores as opposed to formation of cyclobutane pyrimidine dimers in vegetative cells. Repair of these photoproducts is an important factor when treating bacterial endospores with UV disinfection techniques. Currently, it is known that endospores possess two types of repair mechanisms 1) nucleotide excision repair which is similar to the damage repair system present in vegetative cells and 2) SP-specific repair, a mechanism for splitting SP in DNA to two thymines without cleaving the DNA backbone. These repair systems operate in the early stages of spore's germination and the activity of these repair systems has been studied for UV inactivation at 254 nm [Donnellan and Stafford, 2006]. However, there is a lack of data on the UV repair potential of pulsed UV treated endospores. Research indicates that the presence of certain proteins (α/β-type SASP) within the spore structure also result in the resistance of bacterial spores to many methods of disinfection including heat, chemical and monochromatic UV [Setlow, 1968]. There are high levels of this protein present in the *Bacillus* species including *B. cereus* and *B. megaterium* and binding of this protein to DNA has been linked to disinfection resistance [Setlow, 2001]. The findings of this study also suggest that endospore resistance to disinfection with pulsed UV light is not affected by the pathogenicity of the *Bacillus* species studied. Indeed similar levels of inactivation were obtained for the non-pathogenic and pathogenic strains studied (Table 2).

Studies have shown that once microbes are entrapped in particles or absorbed to surfaces, they can be shielded from disinfection [Verhille, 2003]. Therefore, it is reasonable to assume that bacterial endospores would be more resistant in an actual water treatment facility due to their aggregation properties. At present studies focusing on the UV inactivation of organisms is primarily based on bench-scale inactivation kinetics based on laboratory studies using primarily dispersed suspensions of organisms. This highlights the importance of conducting plant scale studies on problematic pathogenic organisms such as *Cryptosporidium*. However, there are limitations to the extent of pulsed UV studies which can be conducted at plant level due to the

pathogenic nature of *Cryptosporidium* species. This has prevented more in depth studies focusing on the implementation of the pulsed UV light system at operational plant level.

The findings of this study show that the non-pathogenic organism *B. megaterium* has similar inactivation rates to the parasite *Cryptosporidium parvum* ($p < 0.05$) following exposure to pulsed UV light at 16.2 J. Therefore, *B. megaterium* may allow for future inactivation studies on the optimization of the PUV system at water/wastewater treatment plant level by providing a surrogate organism for the PUV inactivation of *C. parvum*.

In conclusion, the findings of this study showed that pulsed UV light effectively eliminated high levels of waterborne parasite oocysts from *Cryptosporidium* and recalcitrant endospores after extend pulsing intimating that it is a potential application for use at WWTP level.. Findings also suggest that the non-pathogenic endospores of *Bacillus megaterium* exhibited greater or similar inactivation rates to oocysts of *Cryptosporidium parvum* following pulsed UV disinfection. Therefore, *B. megaterium* may provide a suitable indicator organism for the pulsed UV inactivation of *Cryptosporidium* oocysts at water treatment plant level. This would allow for full scale inactivation studies at plant level using a non-pathogenic organism which is representative of the pathogenic *Cryptosporidium parvum*. Furthermore, it is recommended that studies into the potential of *Bacillus* endospores to repair genetic damage following pulsed UV treatment be investigated.

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References

Amman, R. R., B. J. Binder, R. J. Olsen, S. W. Chisholm, R. Devereaux, and D. A. Stahl. 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied Environmental Microbiology* 56:1919–1925.

Craik, S.A., Weldon, D., Finch, G.R., Bolton, J.R. and Belosevic, M. (2001) Inactivation of *Cryptosporidium parvum* oocysts using medium- and low-pressure ultraviolet radiation. *Water Research* 35, 1387-1398.

Donnellan, J. E., and Stafford, R. S., (1968) The ultraviolet photochemistry and photobiology of vegetative cells and spores of *Bacillus megaterium*. *Biophysical Journal* 8

Facile, N., Barbeau, B., Vostm M. P., and Koudjonou, B., (2000) Evaluating bacterial aerobic spores as a surrogate for *Giardia* and *Cryptosporidium* inactivation by ozone. *Water Research* 34, (12) 3238-3246

Garvey, M., H. Farrell, M. Cormican & N. Rowan, 2010: Investigations of the relationship between use of in vitro cell culture-quantitative PCR and a mouse-based bioassay for evaluating critical factors affecting the disinfection performance of pulsed UV light for treating *Cryptosporidium parvum* oocysts in saline. *Journal of microbiological methods*, **80**, 267-273.

Keegan, A.R., Fanok, S., Monis, P.T. and Saint, C.P. (2003) Cell culture-Taqman PCR assay for evaluation of *Cryptosporidium parvum* disinfection. *Applied and environmental microbiology* 69, 2505-2511.

Korich, D.G., Marshall, M.M., Smith, H.V., O'Grady, J., Bukhari, Z., Fricker, C.R., Rosen, J.P. and Clancy, J.L. (2000) Inter-laboratory comparison of the CD-1 neonatal mouse logistic dose-response model for *Cryptosporidium parvum* oocysts. *The Journal of eukaryotic microbiology* **47**, 294-298.

Lee, S.U., Joung, M., Yang, D.J., Park, S.H., Huh, S., Park, W.Y. and Yu, J.R. (2008) Pulsed-UV light inactivation of *Cryptosporidium parvum*. *Parasitology research* 102, 1293-1299.

Mazoua, S., and Chauveheid, E., (2005) Aerobic spore-forming bacteria for assessing quality of drinking water produced from surface water. *Water Research* 39:20 5186-5198

Me´ndez-Hermida, F., Ares-Maza´s, E., McGuigan, K., Boyle, M., Sichel, C., and Ferna´ndez-Iba´nˆez, P. (2007) Disinfection of drinking water contaminated with *Cryptosporidium parvum* oocysts under natural sunlight and using the photocatalyst TiO₂. *Journal of Photochemistry and Photobiology B: Biology* 88.105–111

Nieminski, E. C., Bellamy, W. D. & Moss, L. R. 2000 Using surrogates to improve plant performance. *Journal of American Waterworks Association* 92:3, 67–78.

Rochelle, P.A., Marshall, M.M., Mead, J.R., Johnson, A.M., Korich, D.G., Rosen, J.S. and De Leon, R. (2002) Comparison of *in vitro* cell culture and a mouse assay for measuring infectivity of *Cryptosporidium parvum*. *Applied and environmental microbiology* 68, 3809-3817

Rochelle, P.A., Ferguson, D.M., Handojo, T.J., De Leon, R., Stewart, M.H. and Wolfe, R.L. (1997) An assay combining cell culture with reverse transcriptase PCR to detect and determine

the infectivity of waterborne *Cryptosporidium parvum*. *Applied and environmental microbiology* **63**, 2029-2037.

Rowan, N.J., K. Dean, J. G. Anderson, I.S. Hunter, T. Chaithong (2001). Putative Virulence factor expression by clinical and food isolates of *Bacillus* species after reconstitution in infant milk formulae. *Appl Environ Microbiol*, *67*: 3873-3881.

F. M. Schets, G. B. Engels and E. G. Evers (2004) *Cryptosporidium* and *Giardia* in swimming pools in the Netherlands. *Journal of Water and Health*. 191-200

Setlow, P., (2007) I will survive: DNA protection in bacterial spores. *Trends in Microbiology* *15*, 4

Setlow, P., (2001) Resistance of Spores of *Bacillus* Species to Ultraviolet Light. *Environmental and Molecular Mutagenesis* *38*:97-104

Sharifi-Yazdi, M. K., and Darghahi, H. (2006) Inactivation of pathogenic bacteria using pulsed UV-light and its application in water disinfection and quality control. *Acta Medica Iranica*, Vol. 44, No. 5

Sunderland, D., Graczyk, T. K., Tamang, L., Breysse, P. N., (2007) Impact of bathers on levels of *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts in recreational beach waters. *Water research* *41*: 3483-3489

Verhille, S., R. Hofmann, C. Chauret & R. Andrews, 2003: Indigenous bacterial spores as 20 indicators of *Cryptosporidium* inactivation using chlorine dioxide. *Journal of water and health*, *21* **1**, 91-100.

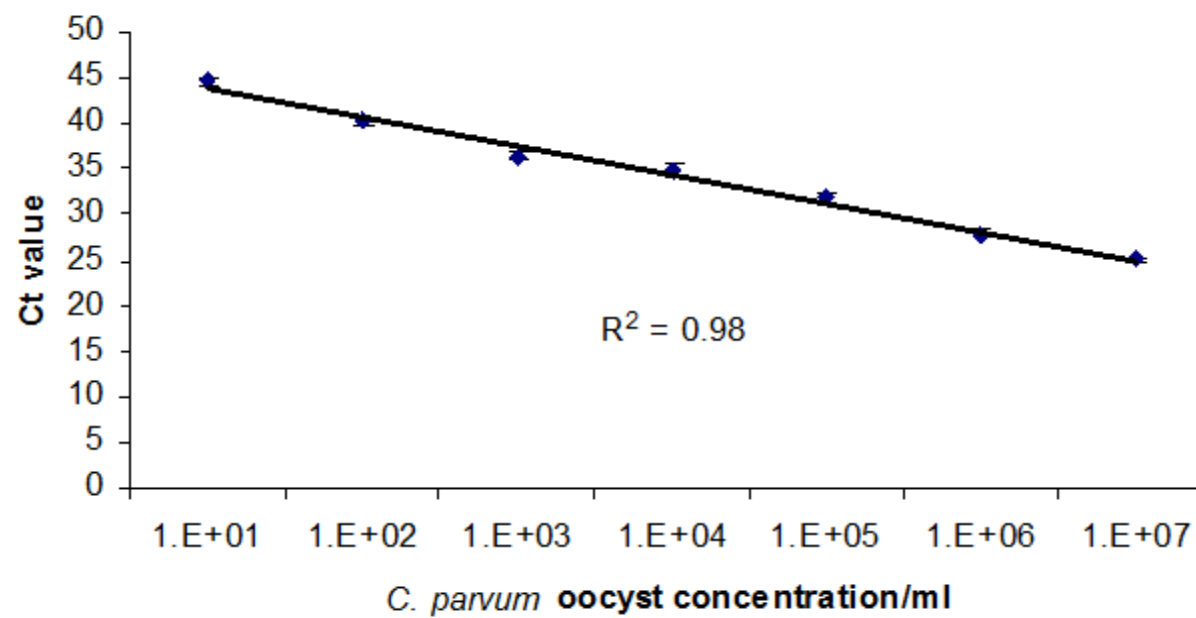


Figure 1. Standard curve for *C. parvum* oocyst concentration versus Ct value obtained by Real time PCR utilising a cell culture based assay (+/-S.D).

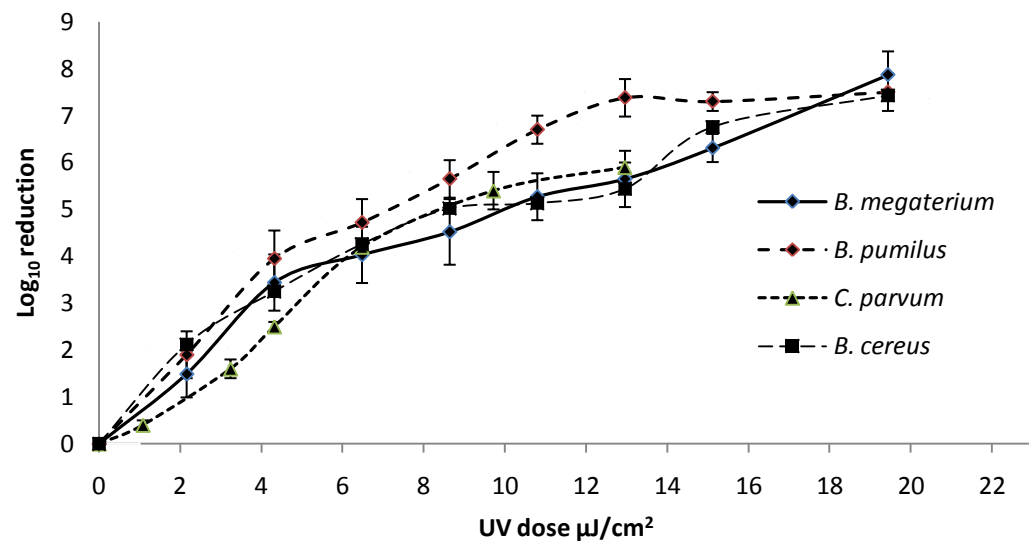


Figure 2. Pulsed UV inactivation of *Cryptosporidium parvum* oocysts and *Bacillus* endospores in PBS containing 10% w/v humic acid at lamp discharge energy 16.2J using a rate of 1 pulse per second

Table 1. Log₁₀ reduction obtained following PUV treatment of varying strains of *Bacillus* species in their vegetative state (+/-S.D) at a rate of 1pps and 1.62J per pulse.

Pulse number 16.2J	UV Dose ($\mu\text{J}/\text{cm}^2$)	Log ₁₀ reduction in microbial test strain		
		<i>B. megaterium</i>	<i>B. pumilus</i>	<i>B. cereus</i>
0	0	0	0	0
10	1.08	3.38 (+/-0.3) A	3.39 (+/-0.4) B	1.77 (+/-0.3) C
20	2.15	5.18 (+/-0.5) A	4.69 (+/-0.2) B	2.81 (+/-0.2) C
30	3.24	5.31 (+/-0.3) A	4.66 (+/-0.5) B	3.06 (+/-0.5) C
40	4.32	6 (+/-0.5) A	4.6 (+/-0.1) B	4.14 (+/-0.1) C
60	6.48	6.5 (+/-0.3) A	4.91 (+/-0.5) B	4.84 (+/-0.3) C
80	8.64	6.7 (+/-0.3) A	6.2 (+/-0.4) B	5.41 (+/-0.1) C
100	10.8	6.62 (+/-0.1) A	6.6 (+/-0.02) A	6.05 (+/-0.5) A
120	12.96	6.9 (+/-0.6) A	6.65 (+/-0.2) A	6.55 (+/-0.4) A
140	15.12	7.1 (+/-0.3) A	6.84 (+/-0.2) A	6.79 (+/-0.5) A
200	21.6	7.2 (+/-0.3) A	7.02 (+/-0.2) A	7.25 (+/-0.1) A

A,B, C denotes significant difference (P<0.05) in strain sensitivity to UV exposure.

Table 2. Log₁₀ reduction obtained following PUV treatment of varying strains of *Bacillus* species endospores and *Cryptosporidium parvum* (+/-S.D) at a rate of 1pps and 1.62J per pulse.

Pulse No.	UV dose $\mu\text{J}/\text{cm}^2$	<i>B. megaterium</i>	<i>B. pumilus</i>	<i>B. cereus</i>	<i>C. parvum</i>
0	0	0	0	0	0
20	2.159	1.49 (+/-0.5)A	1.9(+/-0.5)B	2.1(+/-0.5)C	1.1(+/-0.2)D
40	4.32	3.44(+/-0.6)A	3.95(+/-0.6)B	3.25(+/-0.3)A	2.5 (+/-0.1)D
60	6.48	4.03(+/-0.6)A	4.72(+/-0.5)B	4.26(+/-0.5)A	4.2 (+/-0.1)A
80	8.64	4.52(+/-0.2)A	5.65(+/-0.4)B	5.02(+/-0.6)C	4.9 (+/-0.3)C
100	10.8	5.3(+/-0.3)A	6.7(+/-0.3)B	5.13(+/-0.2)A	5.6 (+/-0.1)A
120	12.96	5.65(+/-0.6)A	7.38(+/-0.4)B	5.44(+/-0.2)A	5.9 (+/-0.1)A
160	15.12	6.31(+/-0.3)A	7.3(+/-0.2)B	6.76(+/-0.5)A	ND
200	19.44	7.87(+/-0.5)A	7.5(+/-0.4)A	7.43(+/-0.1)A	ND

A,B,C,D denotes significant difference ($p < 0.05$) in strain sensitivity or PUV treatment

ND no target DNA detected via qPCR techniques