Studies on the novel detection and destruction of the enteroparasite *Giardia lamblia* and other veterinary problematic microorganisms examined under varying culture conditions

Gillian Coughlan

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Supervised by:

Dr. Noel Murphy (Maynooth University)

Prof. Neil Rowan (Athlone IT)

Dr. Mary Garvey (IT Sligo)Submitted to National University of Ireland Maynooth**,** October 2015

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Abstract

Aims & Rationale:

This study focuses on the hypothesis that every pet entering a hospital environment runs the potential risk of contracting a nosocomial infection. *Giardia lamblia* (*G. lamblia*) is a zoonotic unicellular flagellated protozoan parasite that infects both human and non-human mammals. The presence of pathogenic organisms in veterinary settings is a public health concern in relation to human and animal exposure. Veterinary clinics represent a significant risk factor for the zoonotic transfer of pathogens especially in cases of wound infection and the shedding of faecal matter.

This study aims to provide a means of detecting veterinary relevant parasite species in bacterial biofilms, and to provide a means of disinfecting these parasites and biofilms. This study aims to breach the testing void for *G. lamblia* that currently exists in Ireland by using a combined in vitro HCT-8 cell culture-quantitative PCR assay for evaluating the efficacy of using pulsed UV light for treating *G. lamblia* parasites. This study aims to provide a source of data into novel disinfection mechanisms such as Pulsed UV light technologies as a potential clinical application in veterinary practice.

Methods & Results:

Biofilms were grown on veterinary relevant surfaces, i.e. stainless steel and PVC coupons using a CDC biofilm reactor and treated using Pulsed UV light. Pulsed light successfully inactivated all test species (*Listeria, Salmonella, Bacillus, Escherichia*) in planktonic and biofilm form with an increase in inactivation for every increase in UV dose.

Biofilms were also used as part of a parasite entrapment study for this project. *Giardia lamblia* was seeded in the biofilm reactor and disinfection studies were carried out. In order to independently define inactivation during testing of the novel disinfection methods an *in vitro* cell culture model was adapted and used. A real time PCR assay was utilized to detect parasite

DNA in *Bacillus cereus* biofilms on stainless steel and PVC surfaces. Results show that *Giardia* attach to biofilms in large numbers (100-1000 cysts) in as little as 72 hours.

Conclusion:

This represents the first study on the use of a combined cell culture - real time PCR *in vitro* assay for the viability assessment of low-pressure and pulsed UV light treated *Giardia lamblia* cysts using human intestinal derived cell lines. It is envisioned that such an assay provides an alternative approach to that of *in vivo* testing by allowing for a rapid method of determining parasitic inactivation following UV and other disinfection processes. The observations from these findings further enhance the hypothesis that pulsed UV light would be an effective sterilization technique in a veterinary clinical setting once regular cleaning had taken place. Findings indicated that current *Giardia* detection methods are limited to using vital stains before and after cyst excystation are not appropriate for monitoring or evaluating cyst destruction post PUV-treatments. Use of the human ileocecal HCT-8 cell line was superior to that of the human colon Caco-2 cell line for *in vitro* culture and determining PUV sensitivity of treated cysts. The use of the *in vitro* HCT-8 cell culture assay may replace use of animal models for determining disinfection performances of PUV for treating *G. lamblia.* The extraction and amplification of the parasitic DNA via real time PCR provides a rapid measurement of infective parasite numbers allowing for the measurement of live or dead parasites.

Authors Declaration

I hereby declare that the work contained in this thesis is my own, and was completed with the counsel of my supervisors, Dr Noel Murphy of the Institute of Immunology and Department of Biology, NUI Maynooth, Prof. Neil Rowan of the Biosciences Research Institute Athlone Institute of Technology and Dr. Mary Garvey of the School of Science, Sligo Institute of Technology. The work has not been submitted to any other University or Higher Education Institution, or for any other academic award with this University.

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Gillian Coughlan

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Prof. N. J. Rowan	Dr. M. Garvey	Dr Noel Murphy

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And most importantly, my family. You are my rock and have always encouraged and supported me, and never once waivered. I would be nothing without you.

List of Abbreviations

SS	Stainless Steel
PVC	Polyvinyl Chloride
UV	Ultraviolet
CDC	Centre of Disease Control
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
PCR	Polymerase Chain Reaction
RT-PCR	Real Time Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
WHO	World Health Organisation
µm	Micro meter
ELISA	Enzyme Linked Immunosorbent Assay
SSI	Surgical Site Infection
EPS	Extracellular Polymeric Substances
nm	Nano meter
CPD	Cyclobutane Pyrimidines
PPs	Pyrimidine-Pyrimidone
LP	Low Pressure
MD	Medium Pressure
J	Joules
mJ	Mega Joules
µJ	Micro Joules
W	Watt
MQ	MWga Watt
PL	Pulsed Light

cm	Centimetre
BER	Base Excision Repair
NER	Nucleotide Excision Repair
AP	Apurinic/Apyrimidinic
g/L	grams/Litre
v/v	Volume/Volume
w/v	Weight/Volume
µg	Microgram
PBS	Phosphate Buffer Saline
M	Mole
PI	Propidium Iodide
DAPI	Diamidino Phenylindole
HBBS	Hanks Balanced Salt Solution
RPM	Revolutions Per Minute
°C	Degrees Celsius
CFU	Colony Forming Units

1. Introduction

1. 1. *Giardia lamblia* an emerging zoonotic pathogen

Giardia lamblia (*G. lamblia*) is a zoonotic unicellular flagellated protozoan parasite that infects both human and non-human mammals. *G.lamblia* is considered to be the most common human protozoan enteropathogen worldwide (Zumla *et al.*, 2003) as it is a major cause of gastroenteritis and diarrheal illness, with millions of people being infected every year (Alum *et a*l., 2012) both in the developing and developed worlds. However, the incidence of Giardiasis, the disease caused by the protozoan *G. lamblia*, is generally higher in underdeveloped countries with approximately 15-55% cases occurring annually compared to that of developed countries where an incidence of approximately 5% is seen (Alum *et* al., 2012).

Despite the health significance of its incidence, *G. lamblia* has largely been ignored as a public health threat during the last century and was finally included in the 'Neglected Diseases Initiative' in 2004 (Savioli *et al.*, 2006). Germany made giardiasis a notifiable disease in 2001. The main route of transmission for *G. lamblia* is the faecal-oral route either directly or indirectly. Mechanisms for such transmission include the consumption of contaminated water and food, animal-to-animal, animal-to-human (zoonotic) through the faecal-oral route, through recreational activity such as swimming and human-to-human if strict hand hygiene is not adhered to. Early epidemiological studies which linked giardiasis in campers in Canada with drinking water contaminated with *Giardia* cysts from beavers led to the World Health Organisation (WHO) listing *Giardia* as a zoonosis (Thompson R.C.A., 2004).

1. 1. 1. Taxonomy of *Giardia*

Six *Giardia* species are currently accepted by most researchers; *Giardia agilis, Giardia ardeae, Giardia psittaci, Giardia microti, Giardia muris and Giardia lamblia* (Ryan and Caccio.,

2013). *G. lamblia* is the only species found in humans and it is also found in other mammals including companion animals (cat & dogs) and livestock (Feng and Xiao., 2011). Although there is little variation in the morphology of *G. lamblia*, Ryan and Caccio (2013) reported that there is however at least eight very distinct genetic groups/assemblages (A-H) based on DNA polymorphisms (Table 1.1). Furthermore, within each assemblage there are sub-assemblages based on genetic variation. Isolates that belong to sub-assemblages are genetically close but not identical (Ryan and Caccio., 2013).

Table 1.: The currently recognised assemblages of Giardia lamblia, their host distribution and proposed taxonomy (Ryan and Caccio., 2013).

Assemblage	**Host Distribution**	**Proposed species Name**
A	Humans, other primates, Livestock, Dogs, Cats and some species of wild mammals	*Giaria lamblia*
B	Humans, other primates, Dogs, Cats and some species of wild mammals	*Giardia enterica*
C	Dogs and other canids	*Giardia canis*
D	Dogs and other canids	
E	Hoofed Livestock	*Giardia bovis*
F	Cats	*Giardia cati*
G	Rats	*Giardia simondi*
H	Marine mammals (pinnipeds)	*G. intestinalis*

1. 1. 2. Life cycle of *Giardia*

G. lamblia's life cycle is direct and involves two stages (Ryan and Caccio., 2013); infection and replication. Cysts are responsible for the infective stage of *Giardia,* leading to giardiasis, and the trophozoites (vegetative form), have the responsibility of replication. *G. lamblia* trophozoitess are pear-shaped and appromimately 12-15µm long and 5-9µm wide, while the cysts are approximately 5 X 7 – 10µm in diameter with a wall thickness of 0.3 – 0.5µm (Adam R.D., 2001). *Giardia* is usually a binuclecate organism, in which the two nuclei are apparently equivalent (Kabnick and Peattie, 1990). It is considered that on encystation, the binucleated trophozoite becomes a binucleated cyst, and thereafter each of the two nuclei within the cyst undergoes a single division to form a quadrinucleated cyst (Mayer., 1994). It is assumed that the quadrunucleated cyst establishes the infection in the host (Campbell and Wallis., 2002).

Infection occurs when cysts are ingested through the consumption of contaminated water supplies, contaminated food or fecal-oral route. The acidic pH environment of the stomach provides the necessary stimulation for the cysts excystation and proliferation of the trophozoites in the duodenum (Ryan and Caccio., 2013). Trophozoites then undergo mitotic division in the small intestine causing symptoms such as diarrhea and malabsorption. At this stage, trophozoites may be free moving or attached to the lining of the intestine via their ventral sucking disks. Trophozoites then form cysts in the jejunum after being exposed to bile. These infectious cysts are then excreted in the faeces into the environment, the life cycle being completed once a new host is infected. It is important to note that infected hosts may shed intermittently and therefore repeated samples must be taken before determining if the patient is disease free.

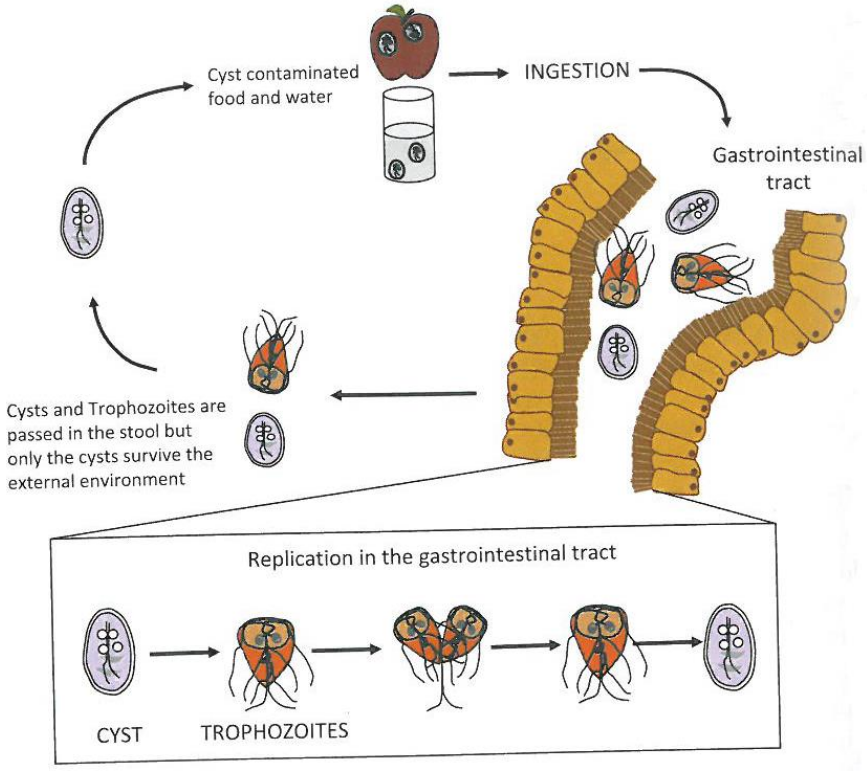


Figure 1.: Giardia life cycle. Image depicting the life cycle of *Giardia lamblia* from ingestion, replication in the gastrointestinal tract and excretion into the environment.(Source: Tracey J Lamb, 2012, Pg 140)

1. 1. 3. Giardiasis

In mammals, including humans, giardiasis is mainly caused by *Giardia lamblia*. In humans, giardiasis is usually a self-limiting illness, characterised by diarrhoea, colic, headache, dehydration, malabsorption, and weight loss (Buret and Cotton, 2011). However hosts are often asymptomatic and shedding the parasite intermittently. Although immuno-competent individuals are not unknown to becoming infected with Giardia, their immune system is usually more adept at fighting and recovering from the infestations. Immuno-compromised, young children and the elderly however, are particularly vulnerable to the parasite. Sanitation, water supplies, population density and general husbandry conditions all affect the rate of infection.

The clinical signs in companion animals include diarrhoea, often light in colour, malodorous and steatorrhoeic, all of which results in poor weight gain and stunting in offspring. Giardiasis may also result in weight loss in adults but rarely in appetence is seen. (Evans, 2005). In Canada, a study investigating the prevalence of Giardia in dogs and cats, vomiting was observed in 17.1% and 16.7% of infected dogs and cats, respectively (Olsen *et al.*, 2010). In agricultural animals, for instance, giardiasis can lead to morbidity and economic losses (Olson *et al*., 2004), although asymptomatic infections are common (Geurden *et al*., 2010).

It has long been argued of the zoonotic potential of *G. lamblia*. Both zoonotic and host specific strains of *G. lamblia* can be harboured in animals (see Table 1.1) and therefore, the importance of molecular analysis tools cannot be over looked, nor can the risk of zoonotic transfer. *G. lamblia* is one of the most common parasites of dogs and cats around the world (Bowman and Lucio-Forster., 2010). A survey in Australia found Giardia cysts in 9.3% of 1400 canine and 2.0% of 1603 feline samples (Palmer *et al.*, 2008). A commercial ELISA (enzyme-linked immunosorbent assay)-based test revealed a positive antigen result in the feces of 15.6% of 16,114 symptomatic dogs and 10.8% of 4978 symptomatic cats in a study carried out in the United States (Carlin *et al*., 2006). 0.3-36% of dogs and cats in Europe have patent infections

with up to 70% of canines being infected in the first year of life (Tenter and Deplazes., 2006). One European study carried out by Sprong *et al.,* in 2009 reported the detection of sub-assemblage AI in 73% of 120 isolates and sub-assemblage AII in the remaining isolates (27%). Veterinarians using ELISA tests from IDEXX Laboratories have witnessed the prevalence of Giardia infections in 'normal' or non-symptomatic cats and dogs to be high: anywhere between 10% to 40%, or higher (Bowman and Lucio-Foster., 2010).

Little is known about *G. lamblia* infection in horses and there is no definitive evidence of the role played by infected horses as potential sources of human infection (Feng and Xiao, 2011), however, in a recent study carried out by SantÍn *et al* on 195 horses in Columbia two horses were identified with assemblage A and 32 horses with assemblage B (Ryan and Caccio, 2013).

A recent study carried out by Geurden *et al.,* (2010) at multiple centres across Europe (Germany, France, Italy and the UK) examining the incidence of *Giardia* in cattle found an overall prevalence of 45.4% (942/2072), with an overall prevalence of assemblage A (43%). The prevalence of assemblage A ranged from 61% in France, to 41% in Germany, 29% in the UK and 28% in Italy. Importantly, 32% of samples had a mixed infection of assemblage A and E.

There are currently no prevalence figures available for *G. lamblia* in Ireland. Testing for *G. lamblia* in-house is a simple ELISA based Snap test commercially available from IDEXX Laboratories. This test which works on an antigen detection mechanism are not ideal however as animals could test positive for Giardia but are not currently infected or shedding into the environment. This in turn may lead to animals receiving antibiotic or anthelminthic therapy unnecessarily. Reference laboratories in Ireland include the Regional Veterinary Laboratories, UCD Veterinary Laboratory and the Irish Equine Centre. However, none of these labs are using PCR based techniques and instead use antigen tests and faecal flotation and staining method.

1. 1. 4. Epidemiology: Human

From the beginning of the 21st century up until 2004, more than 100 waterborne giardiasis outbreaks have been reported worldwide (Plutzer *et al.,* 2010). The largest outbreak of giardiasis, caused by *G. lamblia* in potable water supplies, occurred in Norway in 2004, affecting approximately 1500 people. (Plutzer *et al*., 2010). Of the 45 European countries, only 20 have published scientific research on the prevalence of Giardia in humans and in water samples (Plutzer *et al*., 2010), of which Ireland is not one with published information (see Table 1.2). Due to the lack of countries reporting the incidence of Giardia, and indeed the misdiagnosis of Giardiasis, there are major gaps in understanding its importance. In 2007, a total of 3651 cases of giardiasis were notified to the Robert Koch Institute, Germany (Espelage *et al*., 2010). Approximately 5% of gastroenteritis cases in developed countries are caused by *G. lamblia* compared to that of 15-55% of cases in developing countries (Alum *et al*., 2010). There is an estimated 45,000 reported cases of giardiasis each year in the United States of America (Alum *et al.,* 2010), however, the true incidence is probably higher as cases go misdiagnosed or unreported. Although *Giardia* is not a notifiable disease in Ireland, it is emerging as an issue on pig and dairy farms and could lead to detrimental economic loss if ill thrift amongst young animals is found.

Table 1.: Prevalence of reported Giardia for humans and water supplies in Europe (Plutzer *et al.*, 2010)

Country	Giardia prevelance in human		Giardia prevalance in different water samples and cyst numbers detected in water			
	Symptomatic	Asymptomatic	Sewage water	Raw Water	Surface & Bathing water/swimming pool	Drinking water
Netherlands	5.40%	3.30%			58.6%/5.9% Range: 0-167/10l	
Portugal				15.5%/57.9% Mean: 0-1-108.3/10l		25.40%
Switzerland					97.5% Range 0-216/20l	
Germany	4%			63.8% Range: 0-13143/100l; Average 88.2/100l		14.9% Range: 0-16.8/100l;Average 3.77/100l
Denmark	5.81%	2.97%				
Norway	5.81%	2.97%				
Finland	5.81%	2.97%	Influent 100% Effluent 50%	33.30%	35%	
Sweden	5.81%	2.97%				
Greece					29.6% Range 0-3205/100l	
Hungary	2%		100% Range: inflow 320-5760/l Outflow: 0.6-375/l	48.4%/76.9% Range: 0-1030/100l	33.3% Range: 0-0.8/l	27.2% Range: 0-53.6/100l
Czech Republic				Range: 0-485/100l		
France				84.2%/93.8% Range: 0.5-180/10l	33.3%/67.8%/96.7% Range 0-511.5/10l	
Russia				0-357/2L		
Bulgaria			0-1208/2l		0-232/2l	0-255/2l
Spain			100% Mean influent: 89-8305/l Mean effluent: 79-2469/l	26.9%-55.5% Mean: 1-12.8l	92.3% Mean: 2-400/l Range 0-722/l	19.2-26.8% Mean: 0.5-4/l
Italy			100% Mean 60-7000/l	57.1% Range: 0-8/100l	71% 0.006-80/l	0%
Poland		1-8.8% of healthy children and 3.1-6.5% of healthy adults			2-6.9%	0%
United Kingdom		1.3% of healthy children			Positive	Positive
Albania	17.6% of children	11.19%				
Belgium	4.01%					

1. 2. Nosocomial infections

Nosocomial infections are those that have been acquired in a hospital and specifically relates to one that was not present or incubating prior to the patient's being admitted to the hospital, but occurring within 72 hours after admittance to the hospital. The risk in veterinary hospitals with prevalence studies showing a 4-9% of inpatients acquiring a nosocomial infection (Mielke, 2010) is comparable to that in human hospitals with 5-10% of patients acquiring nosocomial infections (Burke, 2003). Sources of nosocomial agents within the hospital setting includes, but are not limited to, the patient's own flora, staff, and inanimate objects such as instruments, hospital equipment and kennels (figure 2.1). A review carried out by Milton *et al.*, (2015) details both the high risk areas and indeed reasons leading to a heightened risk of acquiring such a disease, these include: veterinary hospitals lacking hygiene, employing invasive devices, prolonged treatment, longer visits by health care worker and caseloads, and lengthy hospital stays. Given several of these risks, it is fair to assume that long-stay, immune-compromised patients hospitalised in a high caseload hospital are at an amplified risk of acquiring a nosocomial disease.

Nosocomial infections can be local infections (e.g., surgical site infection) or area specific (e.g., intensive care unit, neonatal unit). A leading risk of every surgical procedure is the contraction of a surgical site infection (SSI), with 0.8% to 18.1% reporting with complications (Milton *et al.*, 2015). SSI's quite often can be directly related to surgical implants, eg intramedullary pins, valves, joint replacements. This leads to further complications often resulting in the removal of the implant and perhaps the replacement of the implant if required, putting an emotional stress and a financial strain on an owner. Furthermore, the consequences of putting a patient through the physiological strains of unnecessary anaesthesia, surgery and recovery is unethical and is placing the patient at risk. Nosocomial infections are the growing cause of morbidity and mortality in both human and veterinary medicine (Weese, 2008a; Weese, 2008b; Owens

et al., 2008*;* Faires *et al*., 2010), and this therefore could lead to a loss of confidence in the general public and a hospitals client base. Undoubtedly the economic and professional reputation of a hospital and individual practitioner is at stake if an outbreak of a nosocomial disease occurs within a hospital, yet these preventable diseases are somewhat ignored in practice.

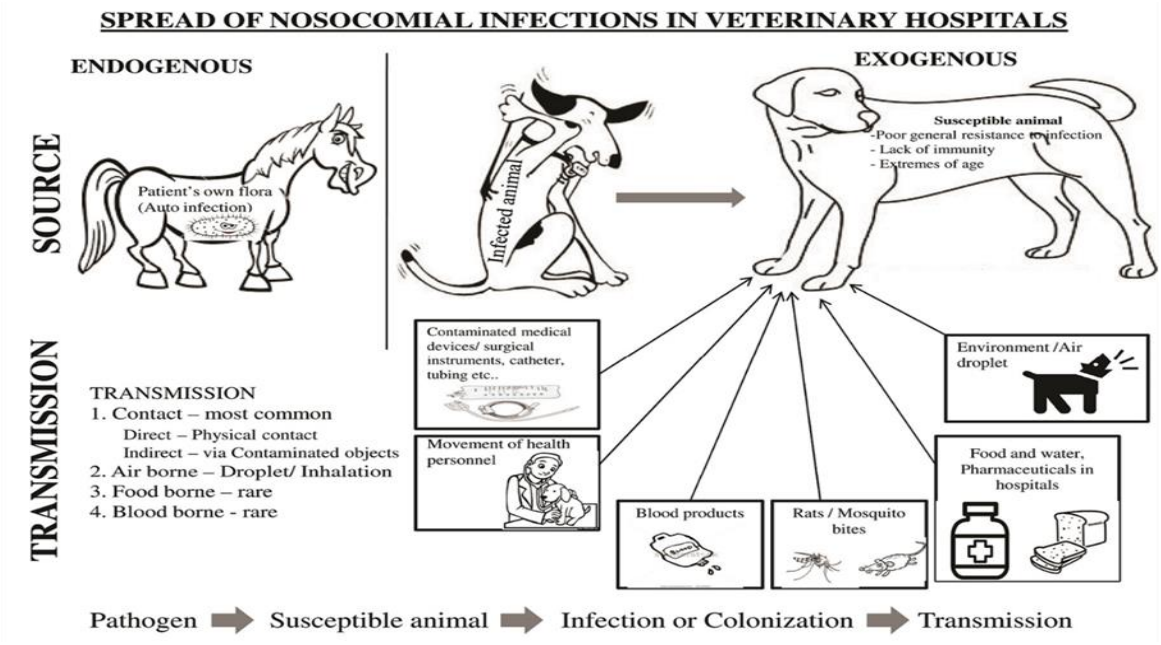


Figure 1.: Spread of Nosocomial Infections within the veterinary hospital (Milton *et al.*, 2015). Image showing sources and transmission routes of infectious agents within the veterinary hospital setting.

1.3 Biofilm structure

Dunne (2002) states that there are three basic ingredients in the formation of a biofilm: glycocalyx, microbes and a surface. And whilst he admits simplifying a rather ingenious technique developed by microbes to aid in their survival in the environment, it is also noted the functional degree of organisation and cooperatively that exists with biofilms to allow maximum interaction with the environment without exhausting resources or compromising cell survival. Carpentier and Cerf (1993) give a slightly more in-depth description of biofilms as "a community of microbes embedded in an organic polymer matrix, adhering to a surface." while Costerton *et al.,* (1999) give the rather proficient description of "a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface." as the definition of a biofilm. In actual fact, a biofilm cannot form if any one of the three components is missing. The terms planktonic (free-floating) and sessile (surface-bound) are the terms used to describe microorganisms. Abiotic surfaces (inanimate) and biotic surfaces (living cells/tissue) are both possible sites of adhesion, colonisation and biofilm formation of microorganisms (Dunne., 2002).

Biofilms are functional complex structures with varying distributions of cells and other essential materials that form a protective environment for the growth and support of vast microbial numbers. These structures can form on to many surfaces including those found in veterinary hospital settings and agricultural processing plants, indeed theoretically there is no surface that cannot be colonised by a biofilm (Bonez *et al.*, 2013). Furthermore, the presence of biofilms in clinical settings and their role in pathogenicity has been well documented (Garvey *et al.*, 2014). Indeed, up to 80% of bacterial infections are linked to biofilms. Microbial biofilms are associated with numerous infections including pseudomembranous colitis (*Clostridium*), osteomyelitis (*Staphylococcus*), endocarditis (*Enterococcus*), and bacteremia (*Enterobacter*) (Clutterbuck *et al.,* 2007).

Biofilms are communities of microorganisms which live attached to surfaces surrounded by a matrix of extracellular polymeric substances (EPS). This extracellular substance is produced by the organisms and includes proteins, nucleic acids, polysaccharides, and amphiphilic polymeric compounds. This matrix is involved in numerous essential processes including attachment to surfaces, cell-to-cell interconnection, quorum sensing, and exchanges between bacterial subpopulations, tolerance, and exchange of genetic material (Harmsen *et al.,* 2010). Bacterial growth and diversity as well as the development of the biofilm matrix depend on several factors including nutrient availability and hydrodynamic conditions (Schwartz *et al.,* 2009).

1. 3. Veterinary relevant biofilms of bacterial and fungal origin

The presence of biofilms on man-made surfaces such as piping, medical equipment, tubing etc. has highlighted their importance in relation to pathogenicity. The formation of biofilms in undesirable places leads to problems in medical, veterinary and industrial environments as these bacterial communities can resist host cellular immunity (phagocytosis), antimicrobial therapy and biocide treatment (Harmsen *et al.,* 2010). The major problem arises however, because the platonkic killing dose is much less than the biofilm killing dose (biofilm killing dose will be between 1000-1500 times higher than planktonic killing dose) (Costerton, 2000). Antibiotics can be used to kill the microorganism causing the disease, and indeed, in the meantime, the patient becomes asymptomatic. However, because the biofilm hasn't been killed, it acts as a reservoir which starts shedding new bacteria, and thus, the illness 'returns'. Studies by Momba *et al.,* (2000) stated that for each planktonic bacterial cell detected there may be up to 1000 organisms present within a biofilm. It is well known that sessile bacteria are more resistant to treatment with antimicrobial compounds, metal toxicity, acid exposure, dehydration and phagocytosis than planktonic cells (Lindsay and Holy, 2006). Hydrogen peroxide, common household bleach, is effective at killing biofilms by dissolving the

polysaccharide matrix (Costerton, 2000), however, bleach is highly irritant to animal and human mucosa and is toxic to most, therefore the use of bleach on surfaces, feed/water bowls, and agricultural plant pipes etc should be avoided. The resistance of these structures to common disinfection agents such as chlorhexidine means that alternative decontamination methods need to be established.

In the last 3 decades fungi have appeared as a major cause of human disease, predominantly among immunocompromised individuals, neonates, burn patients and patients with serious underlying illnesses (Trofa *et al.,* 2008). *Candida* species are opportunistic eukaryotic fungal pathogens commonly associated with clinical infections resulting in deep tissue infection, high mortality rates and financial burden. *Candida* biofilms are composed of yeast cells and filaments which are structurally attached to biotic or abiotic surfaces and embedded in an extracellular matrix (Nailis *et al.,* 2010). Biofilm formation by pathogenic microorganisms such as *Candida* plays a key role in infections resulting from indwelling devices in the clinical setting. Indeed, the association between *Candida* species biofilm formation and continued host infection has become more evident. Once a biofilms forms, it can continuously supply cells which detach from the main structure into the bloodstream acting as a source of infection. It has been reported by Kumamoto, 2002 that conditions with a high flow rate such as that encountered within the circulatory system may favour the development of persistent biofilms on devices placed in the bloodstream. Furthermore, *Candida* species biofilms are quite resistant to antifungals such as fluconazole, amphotericin B, nystatin and voriconazole (Kumanoto, 2002). In recent years there has been a marked increase in non-*C. albicans* related bloodstream infections from *Candida* species mainly *C. parapsilosis, C. krusei* and *C. tropicalis* (Trofa *et al.,* 2008). The resistance of this species to antifungals and the ability of *Candida* biofilms to tolerate chemical disinfection suggest the need for alternative methods of removing this pathogen from clinical settings. Prevention of infection is a superior method

than infection treatment in terms of cost and patient wellbeing. An alternative or supplementary means of control is to minimise the extent of exposure of the patient to these fungal pathogens, thereby preventing an infection from occurring. Typical clinical surfaces such as plastics have been shown to act as reservoirs for viable pathogenic fungi such as *C. albicans* and *C. parapsilosis* (Neely and Orloff, 2001). Proper cleaning regimens that include the use of effective surface decontamination techniques can help prevent patient exposure to pathogenic species.

The prevention and control of veterinary related infections is an important aspect of public health and safety due to the occurrence of zoonotic infections. The spread of pathogenic species within veterinary practices can lead to infection of both the housed animals and veterinary staff. Veterinary clinics are a connection of human and animal interaction, often in situations dealing with infected wounds or faecal matter. This is a significant concern for immunocompromised individuals who are animal owners. The Department of Health, Queenstown, Australia developed 'The Animal contact guidelines – reducing the risk to human health' in 2014 and has outlined excellent guidelines and information for people visiting/working with animals to reduce the risk of contracting a zoonotic disease. Suffice to say that education and training of veterinary staff, and demanding the highest possible hygiene standards of employees is imperative to the control and reduction of the risk of zoonotic transfer. Veterinary personnel must adopt a risk based approach when handling sick patients and develop a safe system of work to address any threat to human health. Changing behavioural attitudes such as hand hygiene, the use of isolation facilities, personal protective equipment (PPE), employing stringent barrier nursing protocols and changing uniforms prior to leaving the hospital setting are all small steps to achieving a low risk working environment.

Animal associated pathogens of concern to immunocompromised persons include *Cryptosporidium, Salmonella, Listeria, Bacillus, Escherichia coli, Campylobacter* and *Giardia*

(Grant and Olsen., 1999). Furthermore, many research studies have highlighted the connection between the spread of pathogenic organisms from surfaces to patients (Gebel *et al.,* 2013). Consequently, the use of surface disinfectants for the control of pathogens in clinical and veterinary settings has become important due to the increase in antibiotic resistant microbial species and zoonotic infections. However, issues have arisen where some pathogens have shown resistance to commonly used chemical based disinfectants. Such pathogens include the protozoan species *Cryptosporidium* and *Giardia,* and bacterial biofilm structures. Planktonic microbial cells are able to attach to and colonise environmental surfaces by producing an extracellular polymeric substance (EPS), these adherent (sessile) cells are referred to as biofilms. The descriptive terms sessile and planktonic are used to describe surface adherent and free floating bacterial cells respectively. Veterinary important species such as *Listeria, Escherichia, Bacillus* and *Salmonella* are capable of producing these biofilm structures allowing them to gain resistance to standard chemical disinfection methods. Indeed, biofilms or sessile communities are believed to be the causative agent in diseases such as pneumonia, liver abscesses, enteritis, wound infections and mastitis infections in animals (Clutterbuck *et al.,* 2007). In addition, in hosts with functioning innate and adaptive immune responses, biofilm-based infections are often very persistent and remain unresolved. In fact surrounding tissues often undergo extensive damage by immune complexes and invading neutrophils when trying to eradicate the infection (Stewart and Costerton, 2001).

The prevention of biofilm formation would provide the best control measures for these robust structures; however, there is no agent available that will prevent cell adhesion and biofilm formation. Current methods rely on the use of disinfection agents and regular cleaning of surfaces exposed to possible pathogens. Research has indicated that sessile communities can be up to 1000 times more resistant to chemotherapeutics such as chlorhexidine than their planktonic counterparts. Furthermore, resistant bacteria originated in sessile communities can

spread from animal to animal through veterinary staff, veterinary surfaces and equipment or farm equipment such as feeders and water dispensers (Aguilar-Romero *et al.,* 2010) resulting in extended infection problems. Biofilm structures are also capable of trapping or incorporating other pathogenic species including viruses and parasites such as *Giardia* and *Cryptosporidium* (DiCesare *et al.*, 2012). Harbouring of such species shields them from cleaning and disinfection techniques, increasing their already high resistance to such treatments. Studies have shown that biofilms represent a significant, long-term reservoir for pathogens such as *Cryptosporidium* and *Giardia* which can be released back into the environment, thus, explaining the presence of parasites in water networks long after disinfection protocols are completed following an outbreak. Indeed, the continued presence of *Cryptosporidium* in a drinking water system following an outbreak in England was attributed to the presence of biofilm structures on the piping network (Wingender and Flemming, 2011). Such findings indicate that alternative ways of pathogen inactivation in the veterinary setting must be provided. Ultraviolet (UV) light is well known for its antimicrobial activity, due to its bacteriostatic properties preventing bacterial cell replication.

1. 4. Ultra-Violet (UV) Light as a disinfection tool

For many years, disinfection of surfaces such as surgical theatres, dental equipment etc has been done using many techniques including chemical, heat and continuous UV light. Residues from many of the chemicals used in disinfection in veterinary practices can leave residues, some of which can be irritant to the user and indeed inpatients hospitalised in these kennels, e.g. bleach. Currently there are many health concerns in relation to the long-term effects of using chemicals as disinfection agents and as water treatment agents. Some microorganisms have become highly resistant to the chemicals used for disinfection, including chlorine e.g. *Cryptosporidium.* Also, high levels of chlorine in potable water can leave an unpleasant taste on the water and so the necessity to use an alternative method of disinfection is required.

In the search for a disinfection method other than chemical, especially in potable water supplies, UV treatment is increasingly looking like the solution to this quest. Clancy *et al* (2000) states, "UV light is considered a viable treatment technology because it has been shown to effectively inactivate pathogens". UV treatment forms limited disinfection by-products (Peldszus *et al*., 2000). It is generally known that DNA molecules absorb UV photons in a range of 200 nm to 300 nm, with the peak absorption at 260 nm. The damage caused to the DNA from the absorption of the UV light alters nucleotide base pairing linkages. If the organism is not capable of photo repair synthesis, the damage goes un-repaired thus resulting in cell death.

1. 4. 1. Ultraviolet light

Ultraviolet radiation/light lies between visible light and X-rays on the electromagnetic spectrum. Ultraviolet light (UV) is divided into UVA (400-320 nm), UVB (320-280 nm), UVC (280-200 nm) and vacuum UV (VUV) (200-100) Figure 1.3. UV light is produced by the sun; UVA and UVB are harmful to humans as both may cause sunburn and can lead to the formation of skin cancers. UVA is known to penetrate human skin more than UVB and is linked to premature wrinkling or skin aging. Due to its short wavelength UVC does not pass the ozone layer so it is rarely observed in nature.

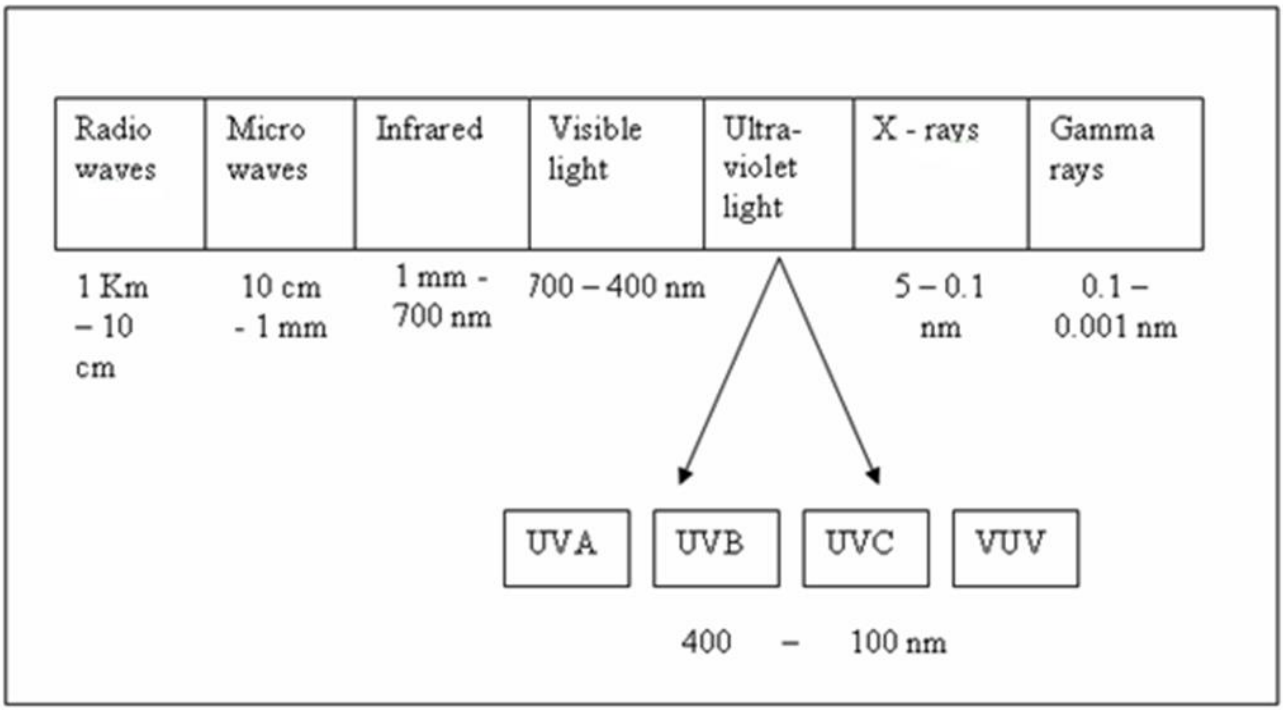


Figure 1.: Electromagnetic spectrum, with the UV region highlighted. Garvey, M. (2009) with permission)

The inactivation of micro-organisms by UV radiation is directly related to the UV dose:

$$Dose = \frac{Time\ (s)\ X\ Output\ (watts)}{Area\ (cm2)}$$

UV dose is the quantity of the energy per unit area that falls upon a surface. UV dose is written as mWs/cm^2. However, UV dose is regularly expressed as millijoules per square centimeter (mJ/cm^2), because 1 mWs = 1 mJ. The UV dose used in water sterilisation in Europe is between 16 and 40 mJ/cm^2 (McDonnell., 2007).

1. 4. 2. UV induced cellular damage

UV inactivates micro-organisms by absorption of light which causes a photochemical reaction that alters the molecular components required by the organism for reproduction. UV radiation penetrates the cell membranes to impact directly on DNA molecules. Nucleic acid absorbs light energy at 240 to 280 nm with an absorption maximum at 265 nm (UVC) (Kiefer, 2007), when DNA or RNA absorbs this energy dimers are formed. The most regular dimers formed are cyclobutane dimers between nearby pyrimidines (CPD) on the same DNA strand, specifically thymine to thymine dimers are most common (Kiefer., 2007) that is instead of pairing with adenine, a thymine base pairs with another thymine (in RNA uracil pairs with another uracil). The thymine dimer forms a four membered cyclobutyl ring, which inhibits DNA replication and function. In total there are three types of pyrimidine dimers: thymine – thymine, thymine – cytosine and cytosine – cytosine. Thymine dimers are more often produced because thymine has a greater absorbance than cytosine in the germicidal range and the quantum yield for the formation of thymine to thymine dimers is greater than that for the formation of the other dimer possibilities (Giese and Darby, 2000). Higher doses of UV light also cause protein damage leading to a loss of structure and function and also can result in cell lysis (McDonnell, 2007). UV energy is absorbed by proteins at 280 nm and there is some

absorption by the peptide bond within protein structures at 240 nm. Additional important biological molecules with unsaturated bonds e.g. hormones, coenzymes and electron carriers may also be vulnerable to destruction by UV. This is an important factor in larger organisms such as fungi and protozoa. Treatment of bacterial spores with UVC leads to the development of the "spore photoproduct" 5-thyminyl-5, 6-dihydrothymine, single and double strand breakage as well as CPD formation (Gomez-Lopez *et al.*, 2007).

Another damage type results from covalent linking between two pyrimidine bases involving the 6-position and the 4-position of the ring, this damage is referred to as "6-4-photoproducts" or 6-4 pyrimidine-pyrimidone (6-4 PPs) adducts (Kiefer., 2007). The frequency with which these 6-4-photoproducts are formed depends on the base composition of the DNA. In *E. coli* lacI and lacZ genes cyclobutane pyrimidine dimers and 6-4 pyrimidine photoproducts form in a 2:1 ratio following UV exposure (Beggs, 2002).

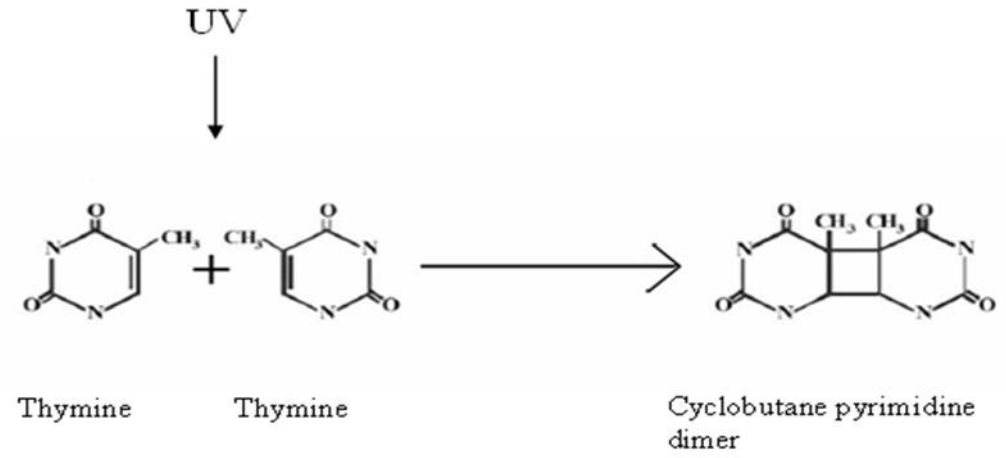


Figure 1.: Formation of CPD as a result of UV exposure. DNA stability and integrity can be interfered with using ultraviolet radiation resulting in DNA lesions such as Cyclobutane-Pyrimidine Dimers (CPD's) (Rastogi *et al.*, 2010)

1. 4. 3. Artificial sources of UV light

The earth's atmosphere prevents UVC also known as germicidal UV from reaching the earth's surface. For this reason for disinfection purposes artificial sources of generating UVC are needed. Producing UV radiation requires electricity to power UV lamps. A UV lamp consists of a quartz tube which contains an inert gas (e.g., argon) and a small amount of liquid mercury. Ballasts control the power to the UV lamps. When a voltage is applied to the lamp, some of the liquid mercury vapourises. Free electrons and ions then collide with the gaseous mercury atoms, "exciting" the mercury atoms into a higher energy state. The excited mercury atoms return to their ground (normal) energy state by discharging energy as UV light. Mercury is favourable for UV disinfection because it emits light in the germicidal wavelength range (200 – 300 nm). The UV light produced depends on the concentration of mercury atoms in the UV lamp, which is directly related to the mercury vapour pressure. UV disinfection uses either low pressure (LP) lamps at a wavelength 253.7 nm or medium pressure (MP) lamps at wavelengths from 180 to 1370 nm or lamps which emit high intensity pulses of light. There are numerous sources of UV radiation, however the most common is the electric arc and mercury lamp which provide continuous sources of UV light.

1. 4. 4. Continuous wave UV

Inactivation of organisms with continuous wave UV light is performed by using low-pressure (LP) mercury lamps designed to emit light at 254 nm i.e. monochromatic light. Due to the distinct disinfection method of UV (the absorption of UV energy at 254 nm by DNA) traditional UV disinfection systems consisted of Low pressure lamps that produce this monochromatic radiation. However, in the late 1990s medium pressure (MP) UV lamps were introduced because they emit polychromatic light including the germicidal wavelengths (200 to 300 nm) (Gomez-Lopez *et al.*, 2007). There is usually no difference in the disinfection ability

between these lamps. But there are advantages and disadvantages to each. MP lamps have a higher germicidal output than LP lamps, and so require fewer lamps for disinfection. However, LP and MP approach to UV delivery have recognised limitations including possibility of photo-reactivation repair in treated pathogens, poor penetrability, considerable energy usage and possibility that mercy can be leaked to the environment if the lamp is broken.

1. 4. 5. Pulsed power technology and the development of an alternative pulsed UV light approach for surface and water decontamination

Pulsed power technology is best described as an enabling technology that finds application in many areas of physical and engineering research as well as industry and defence. Pulsed power technology requires transient generation of high voltage and high current that in turn results in the generation of large peak powers ranging from Megawatts to Terawatts. Depending upon the application, a pulse generator will deliver a large energy level on a single shot basis (or low repetition rate basis) or alternatively will deliver a modest amount of energy (1-10J) at a repetition rate from 10 to 10,000 pulses per second (Rowan *et al.,* 2008). This repetition rate may be achieved either continuously (towards the lower PRF) or short bursts (towards the higher PRF). 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. As the stored energy is released in extremely short bursts, this enables treatment times to be kept very short (seconds), where the average power requirements are very modest (2-4 kilowatts). During each pulse, very high levels of peak power are generated (10-20 MW), and treatment is achieved using the required number of pulses. Therefore, the power and not the energy does the work. In layman's terms, say a flash-lamp uses 20J of energy per pulse and the energy is dissipated in 1s then the power delivered would be 20 W. However, if the energy were to dissipate in 20 μs then the power would be 1 MW, which is a huge difference in peak power and usability. Thus, this repetitive

switching pulsed-power innovation offers a radical new approach to energy delivery and is geared for post peak oil era. In pulsed UV light technology, stored energy is dissipated through a light source in pulses and disinfection/treatment is achieved through delivery of appropriate number of pulses for given applications.

1. 4. 6. Pulsed UV light

Pulsed light (PL) is a novel, non-thermal method of sterilisation and is produced by storing electrical energy in a capacitor and releasing it as a short high intensity pulse with duration of between 1 μs and 0.1 s (Elmnasser *et al.,* 2007). A modest energy input of a few joules (J) can result in high peak-power dissipation of about 10^7-10^8 W. The electrical energy is applied to a xenon flash-lamp in which the energy ionises the gas to create plasma that expands to fill the lamp. Outer shell electrons are stripped away and intense pulses of UV light are emitted. The efficacy of the pulse system is attributed to the unique effects of high peak power and broad spectrum UV content coupled with the ability to control pulse duration and frequency (Anderson *et al*., 2000). The light produced by the lamp includes broad spectrum wavelengths form UV to near-infrared; during each pulse the system delivers a spectrum that is 20,000 times more intense than sunlight at the earth's surface (Elmnasser *et al.,* 2007). The UV dose can be adjusted by increasing or decreasing the frequency of the pulsing. Preliminary findings from other research groups suggests that pulsed light is effective for killing bacteria, fungi, and viruses and the killing effect is much higher in a much shorter time than with continuous UV treatment (Takeshita *et al.,* 2002).

Therefore, pulsed light (PL) is an approach that has received considerable attention as a strategy for decontaminating food, packaging, water and air (Dunn *et al*., 1997; Gómez-López *et al*., 2007). However, PL technology is also a strong candidate for contact surface decontamination in the healthcare and veterinary setting. This approach kills microorganisms by using ultra short duration pulses of an intense broadband emission spectrum that is rich in

UV-C germicidal light (200-280 nm band). PL is produced using techniques that multiplies power manifold by storing electricity in a capacitor over relatively long times (fractions of a second) and releasing it in a short time (millionths or thousandths of a second) using sophisticated pulse compression techniques (Rowan *et al.,* 1999; Gómez-López *et al.,* 2007). The emitted flash has a high peak power and usually consists of wavelengths from 200 to 1100 nm broad spectrum light enriched with shorter germicidal wavelengths (MacGregor *et al*., 1998; Gómez-López *et al*., 2007). This technology has received several names in the scientific literature: pulsed UV light (Anderson *et al*., 2000; Sharma and Demirci, 2003; Wang *et al*., 2005), high intensity broad-spectrum pulsed light (Roberts and Hope, 2003), pulsed light (Rowan *et al*., 1999), intense pulsed light (Gómez-López *et al*., 2007) and pulsed white light (Marquenie *et al*., 2003). Seminal developments pertaining to these next generation light-flashing technologies has been the subject of recent review (Elmnasser *et al*., 2007; Gómez-López *et al*., 2007), with emphasis strongly placed on decontamination efficacy for food and water applications that aptly reflects the focus of research in this field of study to date. A strong advantage of using pulsed xenon lamps over continuous low to medium pressure conventional UV lamps is that the latter has a characteristic high peak-power dissipation which allows for more rapid microbial inactivation. A continuous 10 W lamp needs to be operated for 10 seconds to achieve the same decontamination efficacy (supplying same energy) as a pulsed lamp of typically 1 MW operated for just 100 µs. Otaki *et al.,* (2003) also reported that adaptive microbial survival (tailing phenomenon) occurs when samples are treated in high turbidity solutions using continuous UV sources, whereas tailing did not occur when similar samples were treated with pulsed xenon lamp.

While current findings from the literature suggests that development of a pulse light approach appears promising, most of the studies to date have focused on food or water applications using a limited range of electro-physical or biological parameters, such as use of

a single lamp discharge energy (J) or fluence (UV dose cm^2) and/or employing a single distance from light source to target treatment area. These landmark *in vitro* and *in vivo* pulsed light studies have been recently reviewed for efficacy in terms of inactivating food-related spoilage organisms and potential microbial pathogens (Elmnasser *et al*., 2007; Gómez-López *et al*, 2007), and include studies carried out using lamp discharge energies of 3 J (MacGregor *et al*., 1998; Rowan *et al*., 1999), 7 J/cm^2 (Gómez-López *et al.*, 2005 and Marquenie *et al*., 2003), 0.99J/cm^2 (Krishnamurthy *et al.*, 2004), 0.7 J/cm^2 (Takeshita *et al*., 2003) and 1 J/cm^2 (Wekhof, 2001). These studies demonstrated that factors such as number of light pulses applied, lamp discharge intensity, distance from lamp to treatment surfaces, shading, microbial species, age and density affected the efficacy of PL decontamination performances.

Table 1.: Discharge voltage and corresponding energy per pulse for the SAMTECH pulsed UV system PUV-01. Garvey, M. (2009) with permission)

Voltage V	400	500	600	700	800	900	1000
Energy J	3.2	5.0	7.2	9.8	12.8	16.2	20

1. 4. 7. Factors affecting UV damage

The germicidal effects of UV are proportional to the dose of energy absorbed by the organism in question. The UV dose needed to inactivate micro-organism's increases with cell size and DNA or RNA content. Although the effectiveness of UV is not hindered by chemical water quality parameters, the presence of suspended solids in the water being treated influences UV disinfection; these particles reduce the amount of UV energy reaching the organisms by absorbing or scattering light. The turbidity of the water being treated can also affect the ability of UV to penetrate the water and can shield micro-organisms from the UV energy. Another factor known to affect UV disinfection is the presence of chemical and biological films on the lamps e.g. a build-up of iron and manganese will lead to staining on the UV light system and will interfere with the UV light transmittance into the water. Cell density and cell aggregation during treatment is an important factor when using light energy for disinfection purposes. Also the sensitivity of micro-organisms to UV radiation may differ depending on which growth phase they are in i.e. lag, exponential, stationary or death phase. Microorganisms are most sensitive to light induced damage in the exponential phase.

Advantages and disadvantages associated with UV disinfection (Solomon *et al*., 1998)

Advantages

- UV is environmentally friendly and does not require the use of dangerous chemicals
- UV is economical as hundreds of gallons of water may be treated
- The effectiveness of UV is independent of factors such as pH, temperature and ionic strength
- UV may not lead to the potential formation of any disinfection by products
- Does not alter the taste or other properties of the water
- UV is compatible with other treatment processes

- UV is effective and quick
- No toxic or irritant residues for patients housed in kennels post treatment.

Disadvantages

Potential disadvantage of using PL technology for surface treatment or water decontamination relate to uncertainties surrounding photo-reactivation and possible repair of treated microorganisms.

Repair mechanisms

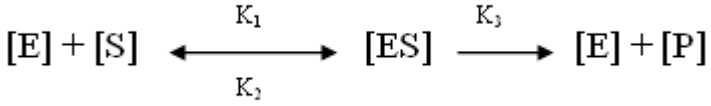
Due to constant exposure to UV from the sun, organisms have developed mechanisms to repair the genetic damage caused by the absorption of photons of UV energy. Conventional UV treatment methods largely affect DNA through mechanisms that are reversible under certain conditions. Micro-organisms have developed two mechanisms for repairing DNA damage caused by the absorption of UV energy referred to as photoreactivation (light) and dark repair.

Photo-reactivation

Photoreactivation is a light dependent repair mechanism. This form of DNA repair involves the use of a single enzyme photolyase which specifically binds to CPDs or 6-4PPs and reverses the damage using the energy of light. This mechanism is a two-step process; the enzyme photolyse combines with the dimer in the absence of light to form an enzyme-substrate complex, this complex is activated by the absorption of a photon of light between 320 and 410 nm and with the additional action of flavin adenine dinucleotide the enzyme splits the cyclobutane ring to restore the original structure (Beggs., 2002). Photoreactivation has been seen to occur in both eukaryotic and prokaryotic organisms. UVA is essential for photoreactivation, although it also causes DNA damage; this is referred to as concomitant

photoreactivation because the light energy originally causing the damage has the potential to repair the dimer.

The kinetics of the photoreactivation process can be represented by the equation:



Where [E] = enzyme, [S] = substrate, [ES] = enzyme substrate complex, [P] = repair product, K_1 = rate constant for production of [ES], K_2 = rate constant for dissociation of [ES] and K_3 is photolytic reaction rate constant (Beggs, 2002).

Organisms including *E. coli* and *C. parvum* are known to possess photolyase enzymes while viruses have no repair mechanisms that can repair UV induced DNA damage (Rochelle *et al.,* 2004).

Dark repair

UV induced lesions other than thymine dimers i.e. cytosine dimers, can only be repaired by dark-repair mechanisms. Dark repair mechanisms replace the damaged DNA with new undamaged nucleotides by a process of excision of the damaged bases from the DNA strand. There are two major types of excision repair; base excision repair (BER) and nucleotide excision repair (NER). Base excision repair is performed with the use of enzymes referred to as DNA glycosylases which remove damaged bases by cleavage of the N-glycosidic bond between the base and the 2-deoxyribose moieties of the nucleotide residues. Different glycosylases remove different kinds of damage. When the base is removed the site which is left (referred to as the apurinic/apyrimidinic (AP) site) is removed by an AP endonuclease or an AP lyase enzyme.

The nucleotide excision repair processes involves the action of proteins that organize the removal of the DNA damage. The enzymes identify and bind to the helical deformation created at the damaged site, and initiate nucleotide excision repair. A repair complex is assembled and cleaves the DNA at positions a few bases to either side of the lesion, leaving a gap. DNA polymerase then replaces the missing DNA using the bases on the opposite DNA strand as a template. Finally, DNA ligase reseals the repaired strand of DNA (Beggs, 2002). Dark repair may occur in UV-exposed drinking water after it is circulated by water supply systems (Morita *et al.,* 2002). Therefore, in order to accomplish an appropriate UV disinfection level, it is essential to quantitatively evaluate the effects of photoreactivation and dark repair in pathogenic organisms (Smith *et al.,* 2005).

Occurrence data from developed and developing countries suggest that *Cryptosporidium* and *Giardia* are commonly found in raw sewage with the latter present in higher numbers more frequently (Garvey and Rowan., 2015). As with parasites such as *Cryptosporidium parvum*, the removal of *Giardia* from water supplies has proven problematic due to its resistance to current water disinfection methods and the low cyst number required for infection to occur.

1. 4. 8. Effects of UV on *Giardia*

Indeed it is due to the emergence of such recalcitrant chlorine-resistant pathogens that the need for alternative water disinfection methods has arisen. The use of UV light technology for the treatment of water has proven effective for numerous water borne microorganisms including parasitic protozoan (Craik *et al.,* 2000, Garvey *et al.,* 2010). The inactivation of microbial species by UV light involves the alteration of DNA following the absorbance of UV energy by the treated species which in turn inhibits the reproductive abilities of the organism. However, studies on the UV inactivation of organisms such as *Cryptosporidium* and *Giardia* are

problematic due to the infective nature of the parasites which require a live host to initiate its reproductive cycle. This coupled with the mode of action of UV light (inducing genetic damage as opposed to cell membrane damage) raises difficulties with accurately determining if *Giardia* has lost its infective abilities following UV exposure.

Although *C. parvum* oocysts are generally considered to be more environmentally robust than *Giardia* cysts, in a study carried out by (Campbell and Wallis, 2002), *G. lamblia* cysts were found to be more resistant than *C. parvum* oocysts to UV irradiation. The same study concluded that a UV dose of 10mJ cm^{-2} results in approximately 2 log inactivation of *G. lamblia* cysts, and a UV dose of above 20mJ cm^{-2} results in up to 3 log (99.9%) inactivation of *G. lamblia* cysts.

Research to date on the UV inactivation of *Giardia* has been based on the use of vital dyes, *in vitro* excystation and *in vivo* infection of live rodents with the former consistently proven to overestimate inactivation (Maux *et al.,* 2002) and the latter raising ethical issues as well as the difficulties and time demands associated with animal testing. The use of an *in vitro* cell culture model as an alternative to *in vivo* testing has proven successful for other parasites such as *Cryptosporidium parvum* (Garvey *et al.,* 2010). Furthermore, as the vital dye viability assay significantly underestimates cyst inactivation as compared with infectivity (Campbell and Wallis, 2002), this suggests that the assay should not be used independently to define inactivation during testing of novel disinfection regimes (Campbell and Wallis, 2002).

By providing an *in vitro* environment similar to that of the host intestines, the parasite can be stimulated to infect cells growing in culture and to initiate its life cycle. *Giardia* trophozoites strongly adhere to the epithelial surface of the intestine via a ventral adhesive disc. A number of parasitic surface molecules are engaged in this tight interaction, including giardins (primarly alpha, beta, delta and gamma giardins), as well as a complex network of contractile proteins which play key roles in trophozoite attachment. The extraction and amplification of

parasitic DNA via real time PCR may then provide a rapid measurement of infective parasite numbers allowing for the measurement of live or dead parasites. Herein, the current study aims to examine the use of a combined cell culture - real time PCR *in vitro* assay for the viability assessment of low-pressure and pulsed UV light treated *Giardia lamblia* cysts using human intestinal derived cell lines. It is envisioned that such an assay may provide an alternative approach to that of *in vivo* testing by allowing for a rapid method of determining parasitic inactivation following UV and other enabling processes for water treatment. Working towards such methods will aid in the treatment and elimination of this pathogenic organism from water supplies by allowing for reproducible studies on the inactivation of *Giardia* cysts for effective water treatment and control.

1. 5. Aims and Objectives

Specifically, this project seeks to:

Aim:

1. Explore the use of a novel surface disinfection approach (pulsed UV light) for the destruction of undesirable parasites, bacteria and fungi in veterinary samples thus avoiding or eliminating subsequent threat of community cross-infections in humans (owners)
2. Complete a comparative study on the Caco-2 and HCT-8 cell lines as infectivity models for *G. lamblia*. Viability via CC-RTPCR compared to *in vitro* excystation assays and dye uptake assays.

Objectives:

- Compare the use of low-pressure UV inactivation of test species to that of the pulsed UV approach both on surfaces and in liquid suspensions.
- Carry out growth and sterilisation of microbial & fungal biofilms on surfaces present in the veterinary environment including a parasite entrapment study within such biofilms

2. Methods

2. 1. Pulsed UV light system

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 in this study as per Garvey *et al.,* (2012). It consists of two main components; a treatment chamber and a driver circuit. The driver unit consists of the trigger and discharge outputs, frequency control, trigger control and the discharge voltage control. The trigger cable connects the trigger output of the 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. This delivery system kills microorganisms by using ultra-short duration pulses of an intense broadband emission spectrum that is rich in the UV-C germicidal wavelength. PUV is produced by storing electricity in a capacitor over relatively long times and releasing it as a short duration pulse using sophisticated pulse compression techniques. 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 which allows it to operate at 1 pulse per second (pps); this setting was used throughout the study. Light exposure was homogenous as the xenon lamp measuring 9×0.75 cm was longer than the 8.5 cm standard diameter.

2. 2. Mammalian cell culture and maintenance of cell lines

Monolayers of the human ileocecal adenocarcinoma cell line HCT-8 (ATCC CCL-244: American Type Culture Collection, Rockville, Md.) were grown with regular sub-culturing 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. Caco-2 cells (ATCC HTB-37), established from a human colon adenocarcinoma Caco-2 cells were maintained at 37°C in Dulbecco modified Eagle's medium/Ham's F-12 medium, supplemented with 20% (v/v) foetal bovine serum, 1% 200 mM L-glutamine, 1% (v/v) non-essential amino acids, 0.5% (v/v) penicillin-streptomycin and 0.5% (v/v) amphotericin B (Sigma-Aldrich). Maintenance media was stored at 4°C and heated to 37°C prior to use. HCT-8 and Caco-2 cells were cultured and maintained in T75 cm^2 cell culture flasks in a humidified incubator at 37 °C in an atmosphere containing 5% (vol/vol) CO_2 for circa. 24 h until 80 to 90% confluent monolayers had formed. Once confluent, cells were trypsinised to remove the cell monolayer from the flask and seeded into 6 well plates for 24 h at 37ºC at a seeding density of 1 $x10^6$ cells/well for use in real time pcr studies and at a density of $1x10^5$ cells/well for chamber slides for infectivity studies using fluorescent stains.

2. 3. Viability and infectivity determination of *Giardia lamblia*

G. lamblia cysts (derived from experimental infected gerbils) were purchased from Waterborne Inc USA. Cysts 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 at 4°C until they were used for UV treatment studies. A combined surrogate dye staining method comprising propidium iodide (PI) 1 mg/ml of 0.1 M sterile PBS, 4', 6'-Diamidino-2-Phenylindole (DAPI) 2 mg/ml in methanol and a fluorescein-labelled mouse-derived monoclonal antibody Giardi-a-Glo™ (having corresponding epitope on cyst cell wall; Waterborne Inc, New Orleans, USA) was used to confirm the viability of cysts. The excystation rate was determined for each batch of cysts by microscopic observation following sequential incubation at 37°C in acidified hanks balanced salt solution (HBSS) for 1 h as per method of Garvey *et al.*, 2010. All experiments were carried out using cysts with greater than 90% viability, as determined by *in vitro* excystation and the uptake or exclusion of vital dyes.

Cysts were counted using a haemocytometer and inverted microscope (Olympus, CKX41) with camera (Olympus, IX2-SLP) attached.

Cell culture infectivity was confirmed by immunofluorescent (IF) staining of treated HCT-8 and Caco-2 cell monolayers following exposure to viable cysts. Cell monolayers were seeded into each of 8 well chambered slides (Lab Tec II, Nunc) at a concentration circa.1×10^5 cells per well. Cysts were stimulated to excyst by re-suspension in acidified HBSS pH 2.7 for 1 h at 37°C. After one washing step with sterile PBS, cysts were re-suspended in cell culture media containing varying concentrations of proteose and thereafter 350 µl aliquots were then added to each well. Samples were incubated for up to 48 h at 37°C in 5% (vol/vol) CO_2 atmosphere, to determine optimal conditions for cell infectivity. At set times each individual well containing a separate monolayer was air dried at room temperature until all moisture had evaporated.Next, 45 µl of Troph-o-Glo™ (Waterborne Inc, UK) which detects different life cycle stages of Giardia *in vitro* was added to each well for 25 mins at 37 °C. Slides were then rinsed from unbound stain by flooding with 100 µl SureRinse (Waterborne Inc, USA). The inoculated 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 to 500 nm and an emission wavelength of 510 to 560 nm for Troph-o-Glo™ and an excitation wavelength of 550 nm and emissions wavelength of 610 nm for the counterstain C101. All wells containing separate monolayers were examined and noted as positive or negative for sites of parasitic infection.

2. 4. UV light treatment of *Giardia lamblia* cysts

Petri dishes used in the tests were placed directly below the lamp source for both pulsed and LP UV (low pressure ultraviolet), which ensured full coverage of the plate surface and eliminated possible shading effects. The LP-UV lamp employed in this study is a handheld

model (UVGL-55) supplied by UVP Inc, UK. It produces radiations in the wavelength range of 254 nm-365 nm, with maximum emission at the germicidal wavelength, 254 nm. For standard treatments the light source was mounted at 8 cm above the treatment area, as this distance was shown previously to be optimal for inactivation of *Cryptosporidium parvum* (Garvey *et al.,* 2010) for both UV methods. In this study, standard treatments involved suspending predetermined numbers of *G. lamblia* cysts in the range of 1 – 1,000,000 cysts/ml in sterile phosphate buffered saline (PBS) pH 7 to pulses of UV light. Samples were treated in petri dishes that were then subjected to lamp discharge energies of 16.2 J (900 volts) at 8 cm distance from the light source up to and including a fluence of 22.68 $x10^{-3}$ mJ/cm^2 at a rate of 1 pulse per second for PUV studies. The UV dose was adjusted by increasing or decreasing the frequency of the pulsing. In order to ensure that any possible negative effects of such treatment was solely as a result of a UV induced change in the natural environment of the test species, studies were also conducted on heat inactivated (70^oC) samples, which were prepared in the same manner. Measurement of UV fluence rate ($\mu J/cm^2$) at each applied pulse was determined using chemical actinometry as first described by Rahn *et al.,* (2003), with the modifications of Hayes *et al.,* (2012) as the non-continuous emitted spectrum did not facilitate use of a calibrated radiometer.

UV dose is reported at mJ/cm^2 for comparative analysis to that of LP-UV. LP-UV inactivation was conducted with a hand help lamp (UVGL-55 handheld UV Lamp) placed 8 cm above the treatment dish, UV dose (mJ/cm^2) was varied by increasing or decreasing the exposure time as required. All studies were conducted in an aseptic environment. Following treatment, treated and untreated controls were viability assessed by fluorescent staining using the method previously described. Parasites were transferred to sterile centrifuge tubes and centrifuged at 10000 rpm for 15 mins to pellet the cysts. The supernatant was removed and the pellet re-suspended in 1 ml of HBSS pH 7.2 for 1h at 37^oC to initiate cyst excystation. Cell

culture infection and real time PCR was then performed in 6 well plates containing a cell monolayer.

2. 5. Combined cell culture-quantitative PCR (CC qPCR) assay for enumerating viable *G. lamblia*

Real-time, Taqman-quantitative PCR (qPCR) was performed using primers (TIB MOLBIOL, Berlin, Germany) specific for the β-giardin region of DNA. Giardins are filamentous proteins with an alpha coiled helix structure and are a component of the attachment mechanism of *G. lamblia* trophozoites to host cells. 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 cycle (CT) and the smaller will Ct value will be. The Ct value 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. Primers coding for β-giardin were used as per method of Bertrand *et al.,* 2009. The Taqman probe with the following sequence: 5'-FAM TCACCCAGACGATGGA CAAGCCCTAMRA-3 was utilised for this study. Amplification reactions (20 µL) contained 5 µL of sample DNA (0.5 µM of each primer, 0.2 µM of probe) and 15 µL of reaction buffer (Roche Diagnostic, West Sussex, England). Both positive and negative controls were included in RT-PCR to validate the results. DNase–RNase free water was used as negative control throughout.

Cycling parameters were initial denaturation for 10 min at 95°C followed by 65 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 Nanocycler® device (Roche Diagnostics). Large numbers of cycles were used to ensure detection of low levels of infection. On completion of each RT-

PCR run amplification curves were analyzed by Nanocycler software (Roche Diagnostics) and a standard curve of oocyst DNA concentration determined.

DNA standards were prepared from fresh cysts ranging in concentration from 10 to 10^7 cysts/ml by dilution in PBS following standard viable count determinations. Aliquots of cysts at different densities were then stimulated to infect the HCT-8 and Caco-2 cell lines that were seeded into 6 well plates at a concentration of circa. 1×10^6 cells/ml at 90% confluency. The latter cell line stimulation occurred by re-suspension and separate incubations for 1 h in acidified HBSS as previously described. 1 ml aliquots of each concentration range of excysted cysts were re-suspended in appropriate cell culture growth media containing varying concentrations of proteose and added to one well of the 6 well plate. Following 48 h incubation at 37°C in a humidified atmosphere of 5% (vol/vol) CO_2, the cell culture media containing the non-adherent *G. lamblia* and *G. lamblia* which was not internalised was removed by aspiration and discarded. Mammalian cells were then washed with sterile PBS and trypsinised using 1 ml 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 500 rpm for 5 min and re-suspended in 200 µl sterile PBS, thereafter the mammalian cells and *Giardia* cell membranes were lysed using PCR template preparation kit (Roche Diagnostics, West Sussex, England) in order to produce DNA (template) and standard curve following infection in both cell lines. Real time PCR was also conducted on excysted cysts without passage through cell culture to determine the Ct values for serially diluted cysts numbers for comparative analysis. The Ct values for cell culture RT-PCR of each dilution amplified in triplicate were plotted against the logarithm of the starting quantity of cysts. The equation of this standard curve was then used to determine the inactivation of UV treated cysts. The aforementioned cell culture PCR procedure was then repeated to determine infectivity of cysts subjected to varying UV parameters or heating at 70°C for 30 min (negative control). Log inactivation of oocysts (L) is

defined by $L=\log_{10}[Nd/N0]$, where N0 is the initial concentration of cysts and Nd 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).

2. 6. Pulsed light inactivation of veterinary relevant bacterial and fungal species.

Pulsed light inactivation was conducted on test strains *(Listeria monocytogenes* (ATCC 11994), *Bacillus cereus* (ATCC 11778), *Salmonella typhimurium* (ATCC 13311), *Escherichia coli* (ATCC 11775), *Saccharomyces cerevisiae* (ATCC 9763) *Candida parapsilosis* (ATCC 22019), *Candida. albicans* (ATCC 10231), *Candida albicans* (clinical isolate NUIG 6250), *Candida krusei* (ATCC 14243), and *Candida tropicalis* (ATCC 13803))in planktonic form in suspension and on agar surfaces for comparative studies to that of the biofilm communities on PVC and stainless steel surfaces. Planktonic cells of all test strains were treated by PUV for comparative analysis to the sessile cells. For PUV studies of fungal and bacterial strains a single colony of the test strain was aseptically transferred to 100 ml of sterile malt extract broth or nutrient broth respectively followed by incubation at 37ºC for 18 hours at 125 rpm. For surface treatment 100 µl of an appropriate dilution was spread aseptically onto malt or nutrient agar surfaces. Test plates were then exposed to pulses of UV light at 16.2J at varying fluences at a rate of 1 pulse per second as per Garvey *et al.,* (2014) up to a PUV fluence of 12 µJ/cm^2 (treatment time of 120 seconds) for surfaces and 11 µJ/cm^2 (treatment time 100 seconds) for fungal suspensions. PUV studies were also conducted on samples diluted from the 18 hour broth in 20 ml final volumes of sterile PBS at 8 cm from the light source, after which 100 µl of treated liquid was transferred to suitable agar and incubated at 37ºC for 24 hour for all test strains and 30ºC for *C. parapsilosis.*

2. 7. Centre of Disease Control Biofilm Reactor

A biofilm reactor designed by Biosurface Technologies Corp, Bozeman, MT and recognised by the Centre of Disease Control (CDC) for biofilm studies was used in this study. The reactor,

which is capable of generating 24 individual biofilms is comprised of a glass vessel, a magnetic stirrer, and a polyethylene lid which holds 8 interchangeable polypropylene rods. Each individual rod has the holding capacity for 3 coupons, therefore 24 coupons and consequently, 24 biofilms. The purpose of the magnetic stirrer is to provide a continuous flow of nutrients over the colonised surface of the coupons.

Figure 2.: Biofilm Reactor. A system allowing for reproducible conditions for the growth of biofilms on various surface types, e.g. stainless steel, PVC coupons etc.

2. 7. 1. Biofilm Growth

Bacterial strains of veterinary relevance, namely *Listeria monocytogenes* (ATCC 11994), *Bacillus cereus* (ATCC 11778), *Salmonella typhimurium* (ATCC 13311) and *Escherichia coli* (ATCC 11775) were chosen for biofilm formation and pulsed light inactivation studies. All strains were cultured and maintained in nutrient agar and nutrient broth (Cruinn Diagnostics Ltd, Ireland) at 37ºC. Additional fungal strains *Saccharomyces cerevisiae* (ATCC 9763) *Candida parapsilosis* (ATCC 22019), *Candida. albicans* (ATCC 10231), *Candida albicans* (clinical isolate NUIG 6250), *Candida krusei* (ATCC 14243), and *Candida tropicalis* (ATCC 13803) were also used. Biofilms were stimulated to form attached to stainless steel and polyvinyl chloride coupons as per Garvey *et al* 2014 and executed using a biofilm reactor recommended by the Centre for Disease Control. Although glucose was previously found to promote biofilm adhesion and proliferation by Garvey *et al*., 2014 and Seneviratne *et al*., 2013, this study was carried out without the addition of glucose to simulate a true-to-life clinical practice environment. The reactor was sterilised at 121°C for 15 min. The biofilm reactor was seeded using aseptic technique with 1ml of the 12 hour specific microbial culture (ensuring that cells were in the log phase of reproduction) and grown for 72 hrs under rotary conditions (125 RPM) at 37ºC for bacterial strains and 30ºC for fungal strains. Cell counts were conducted to determine the seeding density of the reactor before incubation.

To allow for the enumeration of colony forming units (cfu) per microbial biofilm, all coupons were removed aseptically from each reactor rod and rinsed with sterile phosphate-buffered saline (PBS) to remove any planktonic cells. Biofilms were removed aseptically from each coupon by scraping the coupon using a sterile cell scraper into 10 mL of sterile PBS. Serial dilutions were then made, and the standard plate count technique was used to determine the cfu/ml bacterial population in the biofilm.

2. 7. 2. UV light treatment of veterinary relevant bacterial and fungal biofilms

Coupons were removed aseptically from the reactor rods, rinsed with sterile PBS and transferred to a sterile petri dish under aseptic conditions. Two control coupons, one stainless steel and one PVC, were left untreated. These coupons were submerged in 10 ml of sterile PBS and surface scraped using a sterile cell scraper to remove the untreated biofilms and to allow for the determination of biofilm numbers. All other coupons were exposed to pulses of UV light at 16.2J at one pulse per second set 8 cm from the light source at varying fluences which were obtained by increasing the pulse number. Once treated, coupons were submerged in 10 ml of sterile PBS and surface scraped using a sterile cell scraper to remove the treated biofilms and to allow for the determination of inactivated rates. The liquid was then transferred to a sterile 20 ml container and centrifuged at 3000 rpm for 10 mins to pellet the cells. The sample was then re-suspended and agitated to ensure biofilm dispersion. Serial dilutions were made from the biofilms suspension (down to 10^6) and 100 µl spread on triplicate agar plates (malt extract plates for fungal strains) to determine the cfu/ml of treated samples. Plates were incubated at 37ºC and 30ºC for *C. parapsilosis* for 24 hours. This process was repeated for coupons at varying UV doses to determine the $\log_{10}$ reduction obtained with increasing UV dose. A cell count was also conducted on the media present in the reactor vessel by spread plating technique.

2. 8. Detection of *Giardia* cysts in biofilm structures

The biofilm reactor was seeded aseptically with *Bacillus cereus* and *G. lamblia* cysts of 1ml 1 X 10^6 using the technique outlined in Section 2.7.1. The reactor was then incubated at 37°C for 72 h under rotatory conditions (125 rpm) as these conditions were previously shown to be optimal for the formation of bacteria biofilms (Garvey *et al.*, 2014). After 72hrs incubation coupons were removed aseptically from each reactor rod and rinsed with sterile phosphate-buffered saline (PBS) to remove any planktonic cells. Biofilms were removed aseptically from

each coupon by scraping the coupon using a sterile cell scraper into 10 mL of sterile PBS. The liquid was then transferred to a sterile 20 ml container and centrifuged at 3000 rpm for 10 mins to pellet the cells. The sample was then re-suspended and agitated to ensure biofilm dispersion. DNA extraction as outlined in 2.8.1 was performed and RT-PCR performed as outlined in 2.8.2.

2. 8. 1. DNA extraction from biofilm structures

Scrapped coupons suspended in 10 ml volumes were centrifuged at 800g for 10 minutes to pellet the cells, followed by re-suspension in 200 µl of sterile PCR grade water. Target DNA extraction was conducted for *B. cereus* biofilm suspensions using a Roche DNA extraction kit and HP PCR template preparation kit. as per manufactures instructions (Roche Diagnostics, Roche, Ireland) with both treated and untreated microbial pellets suspended in 200 µl of sterile PBS.

2. 8. 2. Real Time PCR

All primers and probes were sourced from Tib Molbiol, Berlin, Germany. For *B. cereus*, the forward primer ACACACGTGCTACAATGGATG and reverse primer AGTTGCAGCCTACAATCCGAA with the taqman probe sequence F-ACAAAGGGCTGCAAGACCGCG—Q coding for the phaC gene was used as per Nayak *et al.,* 2013. Primers coding for β-giardin of *G. lamblia* were used as per previously described (section 2.5). Both positive and negative controls were included in RT-PCR to validate the results. DNA standards were prepared from fresh bacterial cells and *Giardia* cysts ranging in concentration from 10 to 10^8 cysts/ml by dilution in PBS following standard viable count determinations. All PCR cycle conditions were as previously described (section 2.5).

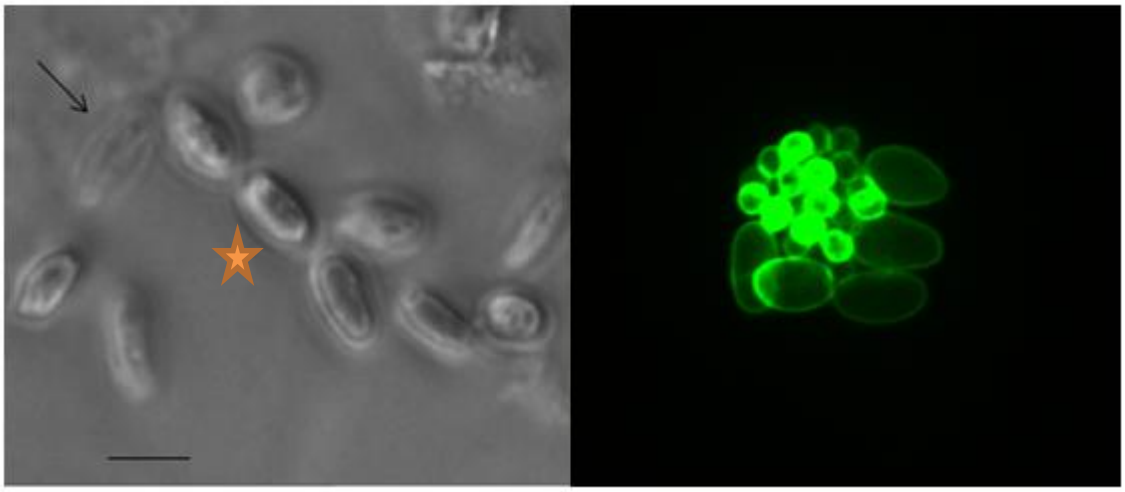
2. 9. Statistical analysis

The log reduction for UV treated cysts was calculated as the $\log_{10}$ of the ratio of the concentration of the non-treated (N_0) and UV treated (N) samples [$\log_{10}(N_0/N)$]. 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 UV treatments. Student t-tests were used to compare infectivity in both cell lines and parasite types. All experiments were conducted in triplicate in three separate experiments.

3. Results

3. 1. Determination of *Giardia lamblia* DNA content via RT PCR

G. lamblia cysts were identity confirmed and viability stained using *Giardia* specific IF dyes (figure 3.1a and 3.1b). Rates of excystation (figure 3.1a) were also checked for all parasite batches before studies commenced. Cysts with greater than 80% excystation rates and 100% viable (figure 3.1) were used for all studies.



(a) (b)

Figure 3.: Image (a) *Giardia lamblia* cysts taken with an inverted microscope; arrow indicates empty cysts structure after excystation (bar 6 µm) while indicates complete cyst. Image (b) fluorescent staining of *Giardia* viable cysts using species specific dyes (Waterborne Inc, New Orleans, USA).

It was found that optimal cell infectivity of the HCT-8 and Caco-2 cell lines occurred in media which contained 2% proteose. The Ct readings for parasitic DNA extracted from cell culture were consistently higher than those extracted from cysts without cell culture indicating that a loss of parasite DNA occurred during this step (figure 3.2 and figure 3.3). Notably, for both cell lines the limit of detection was 10 cysts respectively (figure 3.3) with a detection limit of 1 cyst without passage through cell culture (figure 3.2). These findings were confirmed by the observation of multiple sites of infection via fluorescent microscopy (Figure 3.1.b) and the lower Ct readings obtained following PCR amplification of parasitic DNA (figure 3.3).

Findings show that the human ileocecal HCT-8 cell line was superior to that of the human colon Caco-2 cell line for *in vitro* culture of *G. lamblia* (figure 3.3). This conclusion was drawn based on several observations including: HCT-8 cell line proved significantly more susceptible to infection than the Caco-2 ($p \leq 0.05$), as seen via cell culture infectivity and RT PCR DNA amplification of target DNA (figure 3.3). The negative effect of parasitic infection on host cell lines was more noticeable in Caco-2 cells where cell death occurred more rapidly following exposure to parasites, cells detached from the culture flask following infection of the Caco-2 cells whereas the HCT-8 cells continued to grow vigorously. Taken together these data suggest that the Caco-2 host cell monolayer was unable to support heavy infection rates resulting in a loss of cell viability and attachment, therefore influencing the infection data by producing false negatives.

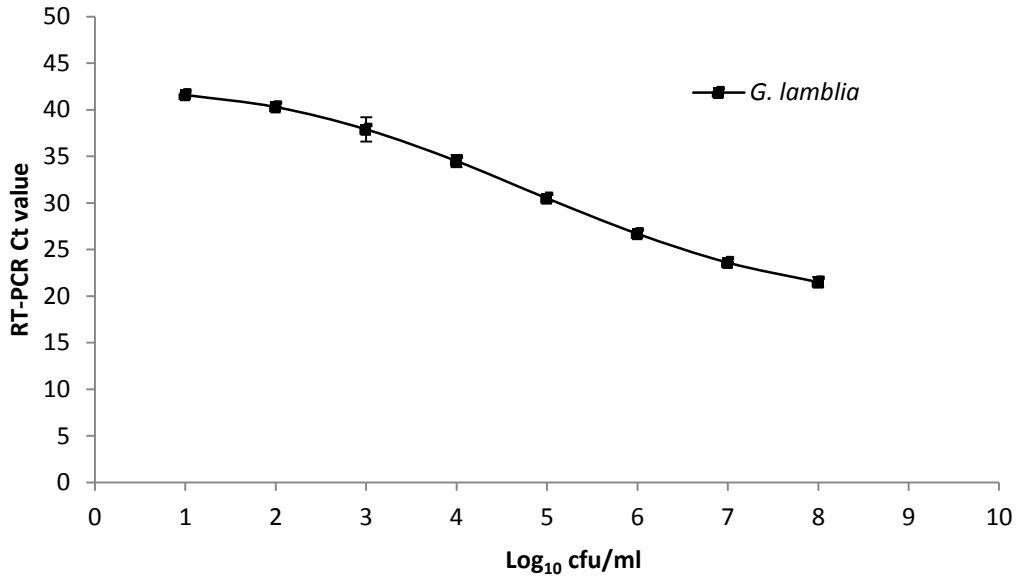


Figure 3.: DNA standard curve as determined by real time PCR analysis for *Giardia lamblia* (log_{10} cfu/ml) (+/-S.D) extracted from cysts without passage through a mammalian cell line using species specific primers.

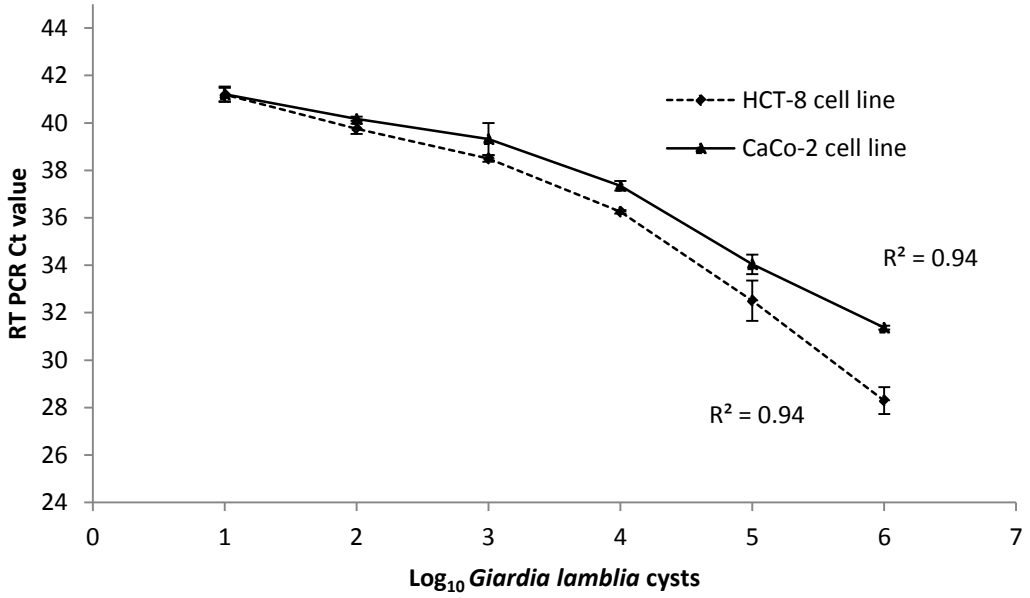


Figure 3.: Standard curve for *Giardia lamblia* infected HCT-8 and Caco-2 cells as detected via real time PCR following 48 hours incubation at 37^{o}C (+\-S.D).

3. 2. Inactivation of *Giardia* with ultraviolet light

In this study, standard treatments involved suspending predetermined numbers of *G. lamblia* cysts in the range of 1-10^6 cysts/ml in sterile phosphate buffered saline (PBS) pH 7 and exposing them to varying fluences of pulsed UV light and LP UV.

Table 3.: Log_{10} reduction and infectivity of treated *Giardia lamblia* via real time PCR and cell culture infectivity IF staining of the HCT-8 cell line following UV treatment (+/- standard deviation).

Exposure	LP-UV			PUV		
time seconds	Dose mJ/cm^2	RT-PCR Log_{10} reduction	Cell culture infectivity*	Dose $x10^{-3}$ mJ/cm^2	RT-PCR Log_{10} reduction	Cell culture infectivity*
0	0	0	4	0	0	4
10	5.4	1.48 (+/- 0.1)	4	1.08	0.52 (+/- 0.1)	4
20	10.8	1.92 (+/- 0.2)	4	2.15	0.52 (+/- 0.5)	4
30	16.35	2.04 (+/- 0.2)	3	3.24	0.6 (+/- 0.2)	4
60	32.7	1.92 (+/- 0.1)	3	6.48	0.62 (+/- 0.1)	4
90	49	1.84 (+/- 0.1)	3	9.72	1.2 (+/- 0.1)	4
120	65.4	1.84 (+/- 0.3)	4	12.96	1.48 (+/- 0.5)	3
150	81.7	1.88 (+/- 0.2)	4	16.2	2.02 (+/- 0.4)	3
180	98.1	1.74 (+/- 0.1)	4	19.44	2.4 (+/- 0.1)	3
210	113.4	1.74 (+/- 0.2)	4	22.68	2.82 (+/- 0.1)	2

*Infectivity of cells as determined by immune-fluorescent staining of 3 separate HCT-8 monolayers exposed to UV treated and untreated *Giardia lamblia* cysts. Presence of infectivity indicates the presence of viable *Giardia.* Numbers indicate level of infectivity 4 = high, 3 = medium, 2 = low, 1 = very low

High = excessive florescent site of infection covering the cell monolayer, impossible to determine numbers due to extreme infection and reproductive stages of life cycle

Medium = visibly reduced number of florescent site of infection. Impossible to determine numbers due to large infection rate and reproductive life cycle stages

Low = visibly reduced level of infection with uninfected parts of the cell monolayer. Impossible to count florescent sites of infection due to large number of life cycle stages infecting localised cells

Very Low = little infectivity noted. Majority of the monolayer uninfected.

Table 3.: Determination of viability of UV treated and untreated *Giardia lamblia* ($1x10^5$ cysts/ml) via staining with vital dyes DAPI and PI compared to the excystation and cell culture RT-PCR assays.

LP-UV dose mJ/cm^2	Viability			PUV dose $\mu J/cm^2$	Viability		
	DAPI/PI (%)¥	Excystation PCR* Log_{10}	CC-PCR^ Log_{10}		DAPI/PI (%)¥	Excystation PCR* Log_{10}	CC-PCR^ Log_{10}
0	100	4.98 (+/-0.1)	5(+/-0.3)	0	100	5.2(+/-0.2)	5(+/-0.05)
5.4	100	5.1(+/-0.01)	3.52 (+/-0.05)	1.08	100	5.1(+/-0.2)	4.48 (+/-0.3)
10.8	99	5.1(+/-0.1)	3.05(+/-0.2)	2.15	100	5.2(+/-0.08)	4.48 (+/-0.1)
16.35	100	5.1(+/-0.08)	2.9 (+/-0.1)	3.24	100	5.2(+/-0.1)	4.4(+/-0.3)
32.7	100	5.0(+/-0.2)	3.08(+/-0.5)	6.48	99	5.2(+/-0.3)	4.38 (+/-0.1)
49.0	99	5.0(+/-0.1)	3.16(+/-0.4)	9.72	100	5.0(+/-0.2)	3.8(+/-0.02)
81.7	100	5.1(+/-0.3)	3.12(+/-0.2)	16.2	100	5.0(+/-0.01)	2.98 (+/-0.2)
113.4	100	5.1(+/-0.1)	3.26(+/-0.3)	22.68	98	4.6(+/-0.1)	2.18 (+/-0.01)

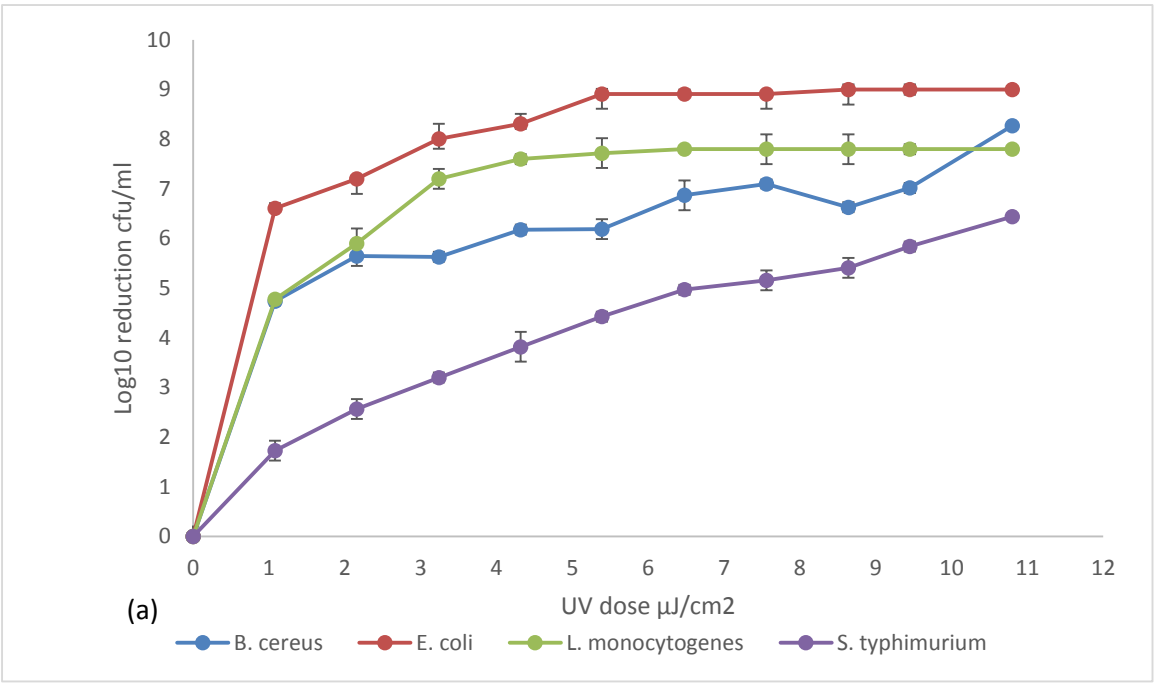
¥ Viability measured by vital staining of *Giardia lamblia* pre and post UV exposure, score is the mean of 3 separate replicates of individual experiments. 100 parasite cysts were counted and marked as positive or negative for dye uptake.

* Log_{10} viability as determined PCR amplification of *Giardia lamblia* UV treated and untreated following excystation without passage through a cell line.

^ Log_{10} viability as determined by PCR amplification of *Giardia lamblia* UV treated and untreated following excystation and cell culture infectivity.

The variance in the Ct values following UV exposure allows for the determination of UV inactivation by comparing treated to untreated controls. The equation of the line obtained for the HCT-8 CC qPCR standard curve was used to calculate $\log_{10}$ reduction of each UV treated batch. Findings show that both LP-UV and PUV light successfully inactivated the test species (table 3.1). With 5.4 mJ/cm^2 of LP-UV dose a 1.48 $\log_{10}$ reduction in viability was achieved for *G. lamblia* as detected via CC-qPCR. With an increase in exposure the rate of inactivation also increased significantly ($p \leq 0.05$) up to a maximum of 1.88 $\log_{10}$. However, after a UV dose of 81.7 mJ/cm^2 no further increase in *Giardia* inactivation was detected (Table 3.1). Therefore, a ca. 2 $\log_{10}$ inactivation rate was obtained with a UV dose of 113.4 mJ/cm^2 equivalent to a treatment time of 210 s (3.5 min). The pulsed UV system resulted in levels of inactivation of *G. lamblia* which were greater than the LP-UV system (table 3.1) with a significantly less UV dose applied. Indeed, a 1.48 $\log_{10}$ inactivation was achieved with as little as 12.96 x 10^{-3} mJ/cm^2 of pulsed UV compared to 5.4 mJ/cm^2 of LP-UV. Noteworthy, the inactivation plateau which was observed with LP-UV did not appear with PUV up to a dose of 22.68 x 10^{-3} mJ/cm^2 equivalent to a treatment time of 210 s. A ca. 3 $\log_{10}$ (99.9%) loss in cyst viability was determined by CC qPCR with a pulsed UV dose of 22.68 x 10^{-3} mJ/cm^2.

3. 3. Pulsed light inactivation of clinical relevant veterinary test species



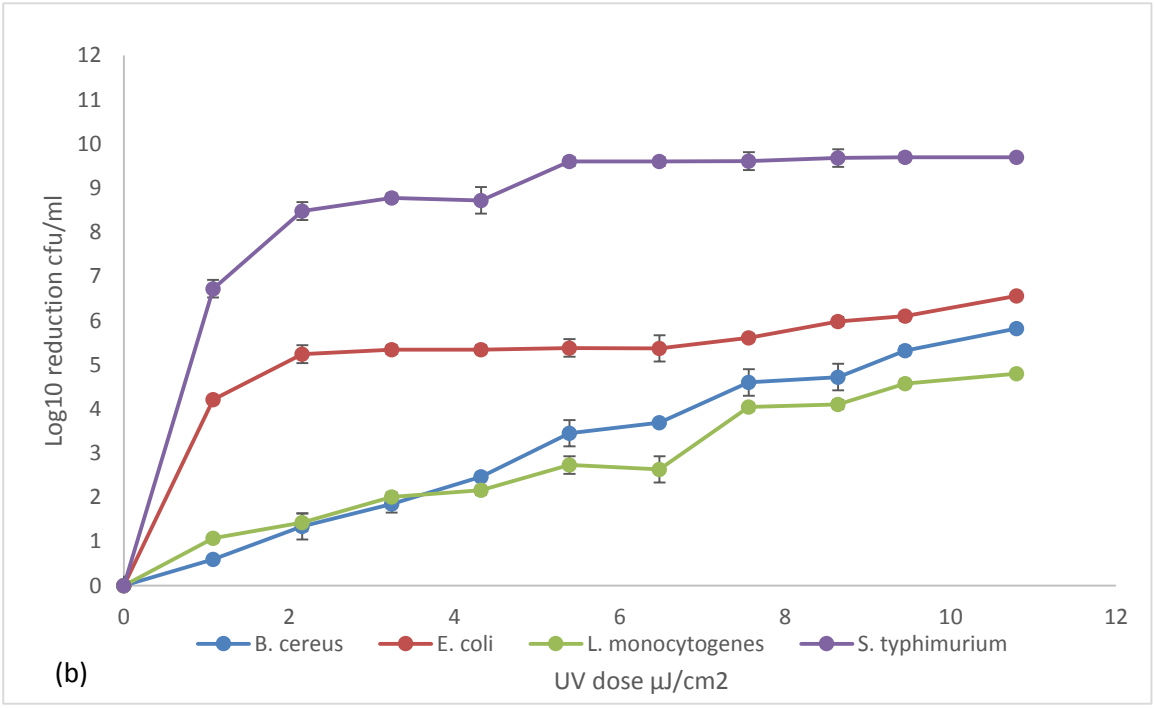


Figure 3.: Pulsed light inactivation of a range of Gram negative and Gram positive bacterial test species on (a) agar surfaces and (b) in suspension (+/- S.D). $\bar{n}$ = the mean of 3 separate replicates of individual experiments.

All test strains proved susceptible to the pulsed light treatment, albeit with varying levels of sensitivity (figure 3.4). *E. coli* showed the greatest level of inactivation on agar surfaces (figure 3.4a) with complete inactivation of an initial concentration of ca. 9 $\log_{10}$ with as little as 5 $\mu J/cm^2$ of pulsed light. The order of decreasing sensitivity for test strains was *E. coli, L. monocytogenes*, *B. cereus* and *S. typhimurium* on surfaces. When treated in suspension this sensitivity changed with *L. monocytogenes* showing the highest resistance to PL treatment and *S. typhimurium* showing the greatest sensitivity to pulsed light (figure 3.4b) for all treatment doses ($p \leq 0.05$). Indeed a maximal 9 $\log_{10}$ inactivation of *S. typhimurium* was achieved with 5.5 $\mu J/cm^2$ compared to a 2.5 $\log_{10}$ for *L. monocytogenes*. This same dose resulted in a 3 and 5 $\log_{10}$ inactivation of *B. cereus* and *E. coli* respectively, highlighting the significant difference in susceptibility to pulsed light

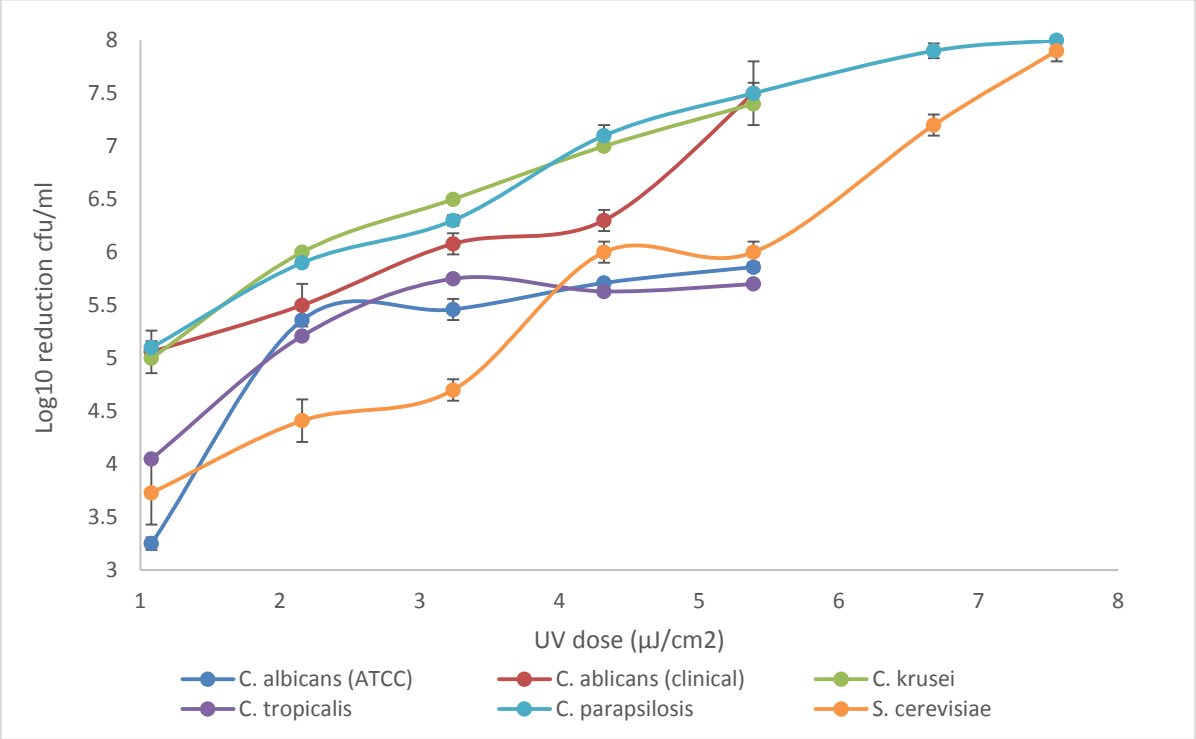


Figure 3.: Pulsed light inactivation (PUV) of *Candida* and *Saccharomyces* test species on agar surfaces (+/-S.D). Π = the mean of 3 separate replicates of individual experiments.

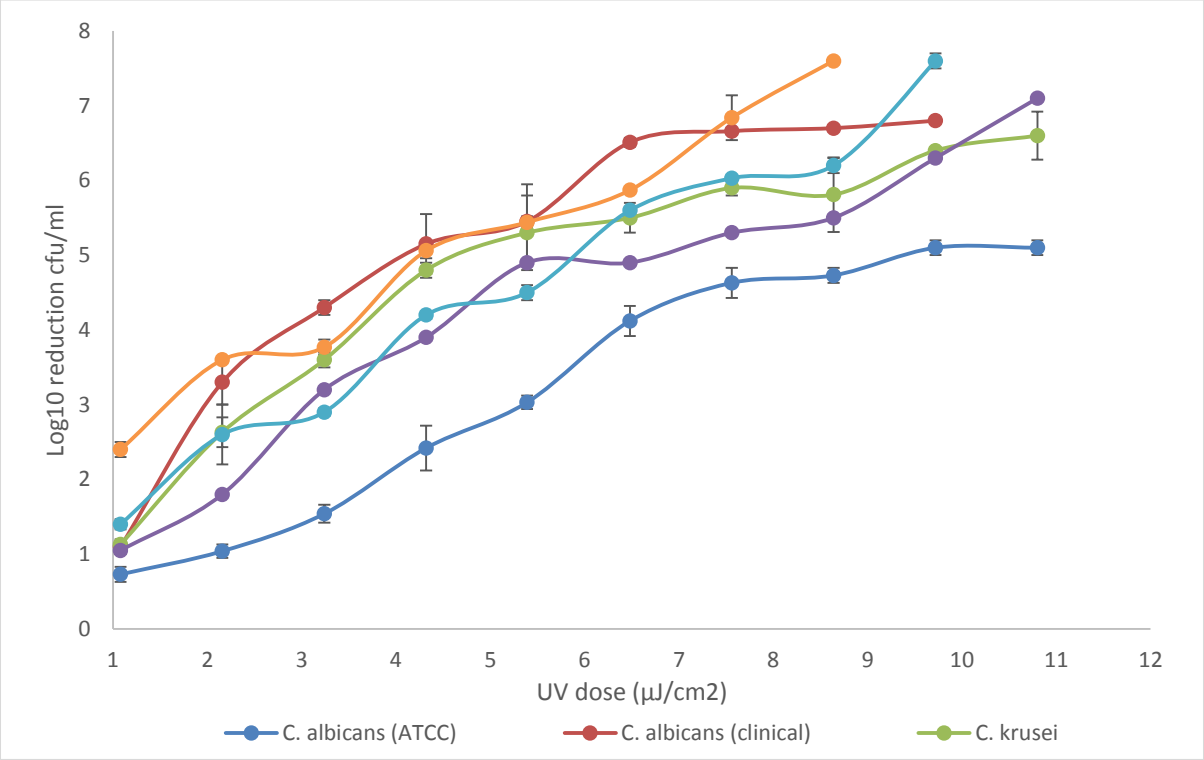


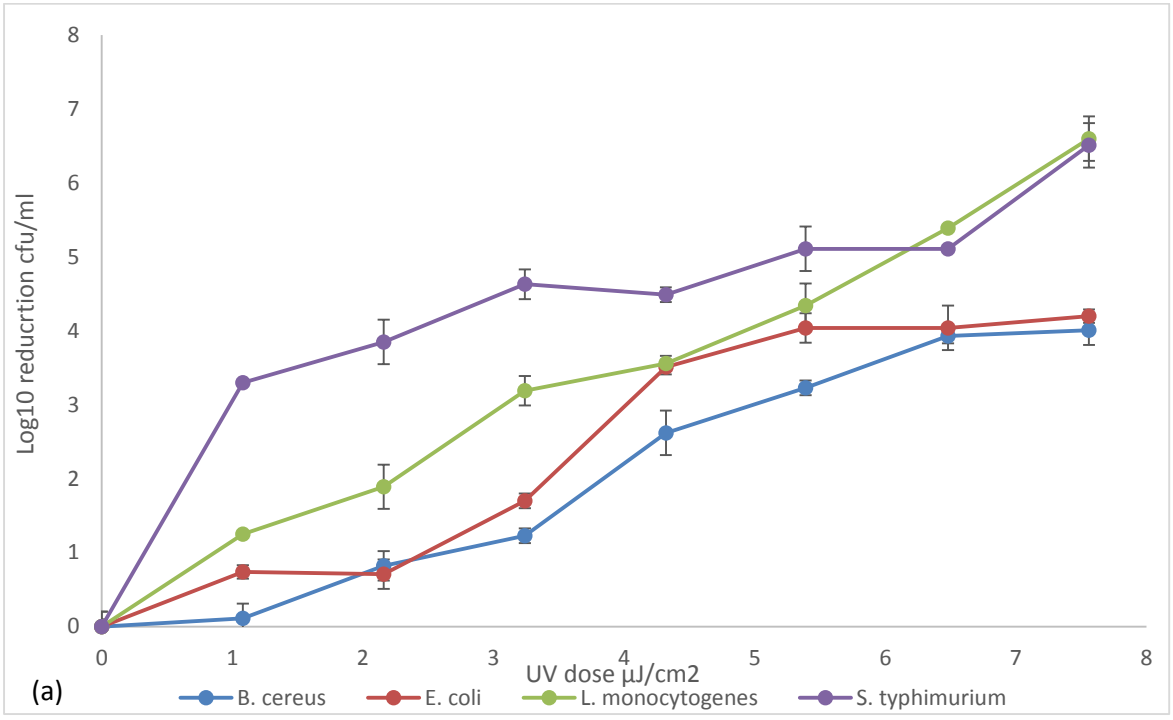
Figure 3.: Pulsed light inactivation of 20 ml fungal suspensions of *Candida* and *Saccharomyces* test strains (+/-S.D). n = the mean of 3 separate replicates of individual experiments.

The *Candida* and *Saccharomyces* test strains under study proved sensitive to PUV inactivation, albeit to varying extents. Figure 3.5 details the inactivation of test strains on agar surfaces following exposure to PUV irradiation. Interestingly *C. albicans* clinical isolate proved significantly ($p \leq 0.05$) more sensitive to UV disinfection that the reference strain *C. albicans* (ATCC) with a 5.6 and 3.25 $\log_{10}$ cfu/ml inactivation obtained with a PUV fluence of 1.08 $\mu J/cm^2$ for each strain respectively on agar surfaces. This trend of an increased sensitivity of the clinical isolate continued for all applied treatment fluences up to 5.39 $\mu J/cm^2$ (treatment time of 50 seconds). A fluence of 5.39 $\mu J/cm^2$ was needed to obtain a 5.8 $\log_{10}$ cfu/ml of *C. albicans* reference strain with 2.15 $\mu J/cm^2$ giving a similar inactivation rate of the clinical isolate (figure 1). *C. albicans* (ATCC) and *S. cerevisiae* showed similar levels of inactivation (ca. 3.2 $\log_{10}$) at 1.08 $\mu J/cm^2$ (treatment time of 10 seconds). This UV fluence resulted in a ca. 5 $\log_{10}$ cfu/ml inactivation of *C. albicans* (clinical), *C. krusei* and *C. parapsilosis* and 4 $\log_{10}$ cfu/ml inactivation of *C. tropicalis*. A fluence of 5.39 $\mu J/cm^2$ resulted in a ca. 5.8 $\log_{10}$ cfu/ml inactivation of *S. cerevisiae, C. tropicalis* and *C. albicans* and a ca. 7.5 $\log_{10}$ cfu/ml inactivation of *C. parapsilosis, C. krusei* and *C. albicans* (clinical). Taken together, these datas suggest that levels of sensitivity to treatment varied with the UV fluence on agar surfaces. The order of sensitivity from least to most resistant to PUV at 5.39 $\mu J/cm^2$ per pulse on agar surfaces was *C. parapsilosis, C. krusei, C. albicans* (clinical), *C. tropicalis, S. cerevisiae* and *C. albicans* (ATCC).

A similar trend was observed when strains were treated in suspension wherein *C. albicans* (clinical) proved more sensitive to UV exposure than the reference strain (figure 3.6) at all treatment fluences. There was no significant difference between the inactivation of *S. cerevisiae* and *C. albicans* (clinical) at treatment fluences of 4.32, 5.39 and 7.56 $\mu J/cm^2$ in suspension with complete inactivation of both strains achieved with 8.64 $\mu J/cm^2$ (figure 3.6). Additionally, *C. krusei* and *C. parapsilosis* showed similar levels of sensitivity to PUV with *C.*

tropicalis proving significantly more resistant than both strains in suspension. Significantly more UV fluence was needed to obtain similar levels of inactivation with fungal suspension compared to surface spread for all test strains. The order of sensitivity from least to most resistant to PUV for fungal suspensions at 7.56 μJ/cm^2 was *S. cerevisiae, C. albicans* (clinical), *C. parapsilosis, C. krusei, C. tropicalis* and *C. albicans* (ATCC).

3. 4. Pulsed light inactivation of clinical relevant veterinary test species biofilms



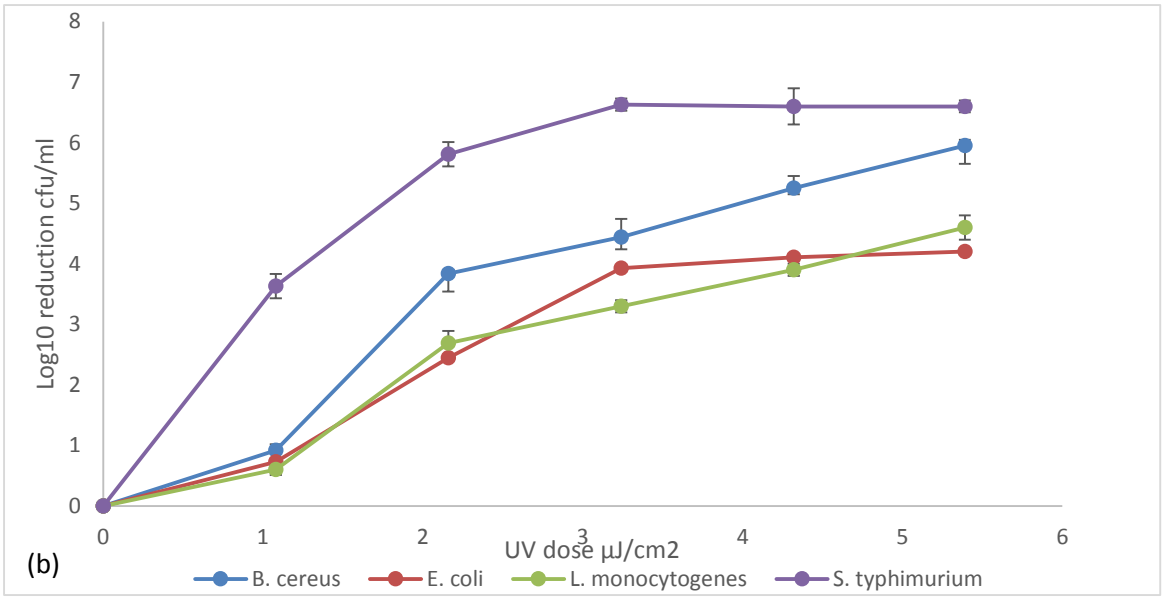


Figure 3.: Pulsed light (PL) inactivation of bacterial biofilms of varying test species on (a) PVC surfaces and (b) stainless steel surfaces (+/- S.D). n = the mean of 3 separate replicates of individual experiments.

All bacterial strains under study formed densely populated sessile communities on both PVC and stainless steel surfaces after 72 hours. Findings also demonstrate that with longer incubation times, exceeding 72 hours (96 hours), there was no increase in cell number of the biofilms as detected by plate counts. Following 72 hours, a ca. 5 and 6.6 $\log_{10}$ biofilm formed for *B. cereus* and *S. typhimurium* respectively, and a 6 $\log_{10}$ for *E. coli* and 6.5 $\log_{10}$ for *L. monocytogenes* on PVC surfaces. A similar level of cell density was detected on stainless steel surfaces, where a ca. 5 $\log_{10}$ to 6.6 $\log_{10}$ biofilm formed for *B. cereus*, *L. monocytogenes*, *S. typhimurium* and *E. coli.*

High levels of biofilm inactivation were also achieved for all test strains present on both surface materials (figure 3.7). For the Gram negative species *E. coli* and *S. typhimurium* a 4.2 and 5.1 $\log_{10}$ reduction in viable cell counts was obtained on PVC surfaces with 5.39 $\mu J/cm^2$ (figure 3.7a). This same dose resulted in a significantly ($p \leq 0.05$) greater level of inactivation of the same species on stainless steel surfaces, with a maximal 4.2 and 6.6 $\log_{10}$ reduction obtained for *E. coli* and *S. typhimurium* respectively (figure 3.7b). Both Gram positive species tested showed increased sensitivity on stainless steel surfaces compared to PVC. A dose of 5.39 $\mu J/cm^2$ resulted in a 3.2 and a 4.3 $\log_{10}$ inactivation on PVC and 5.9 and 4.6 $\log_{10}$ inactivation on stainless steel for *B. cereus* and *L. monocytogenes* respectively. A PL dose of 7.38 $\mu J/cm^2$ resulted in complete inactivation of *L. monocytogenes* and *S. typhimurium* of ca. 6.5 $\log_{10}$ on PVC surface

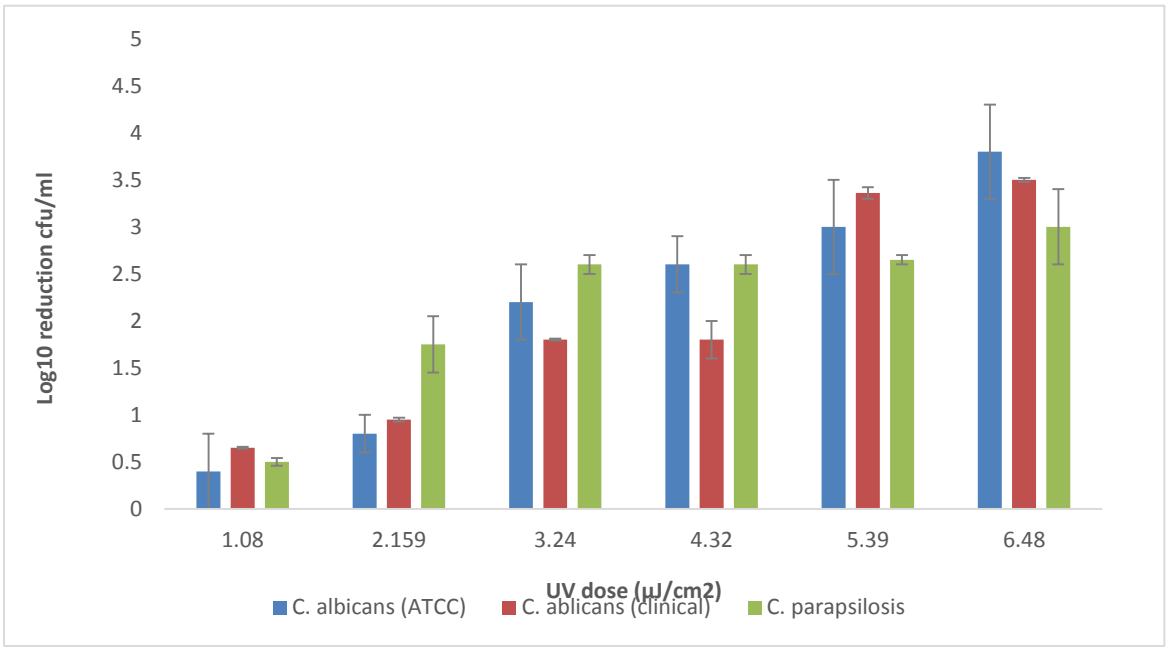
Table 3.: **Clinically relevant Candida species biofilm cell density after 48 and 72 hours growth in a CDC biofilm reactor (+/-S.D).** n = the mean of 3 separate replicates of individual experiments.

	Biofilms cell density (log_{10} cfu/ml)			
	48 hours		**72 hours**	
	PVC	**SS***	**PVC**	**SS***
C. albicans	4.7 (+/-0.2)A	4.2 (+/-0.01)B	4.8 (+/-0.1)A	4.3 (+/-0.05)D
C. albicans (clinical)	4.2(+/-0.1)B	4.2 (+/-0.05)B	5.2 (+/-0.03)F	4.0 (+/-0.1)C
C. parapsilosis	4.2(+/-0.05)B	4.2 (+/-0.1)B	5.1 (+/-0.02)E	5.0 (+/-0.04)E

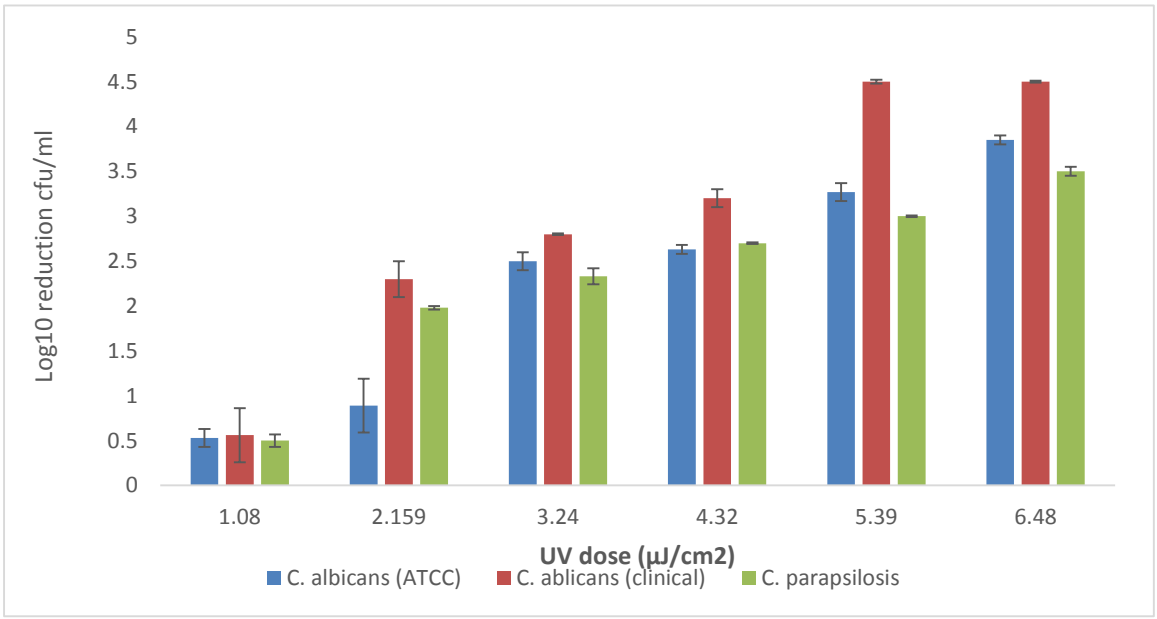
A, B, C, D, E, F denotes significant difference in cfu/ml

*Stainless Steel

The CDC reactor proved an effective tool for the formation and growth of fungal biofilms. Sessile colony counts showed that high density biofilms formed at 48 and 72 hour incubation time points for both surface types in the reactor (Table 3.3). After 48 hours, a 4.7, 4.2 and 4.2 $\log_{10}$ cfu/ml biofilm formed for *C. albicans, C. albicans* (clinical isolate) and *C. parapsilosis,* respectively, on PVC surfaces. There was a significant difference ($p \leq 0.05$) in the sessile population density on stainless steel surfaces where a 4.2 $\log_{10}$ cfu/ml biofilm formed for each test species when compared to that of PVC surfaces. A similar trend was observed following 72 hours incubation with a 4.8, 5.2, 5.1 and 4.3, 4.0 and 5.0 $\log_{10}$ cfu/ml biofilm growths for *C. albicans, C. albicans* (clinical isolate) and *C. parapsilosis* on PVC and stainless steel coupons, respectively. This data suggests that both surface types can support the formation of densely populated *Candida* biofilm structures however; PVC appears more favourable for growth as evidenced by the significantly higher sessile cell count when compared to stainless steel surfaces. Furthermore, it was found that with further incubation (exceeding 72 hours) there was no significant increase in the number of sessile cells present. Biofilm cell counts reached a maximum at 72 hours, after which there was no increase in cell numbers on either surface material, a similar trend to that of Nailis *et al.,* 2010.

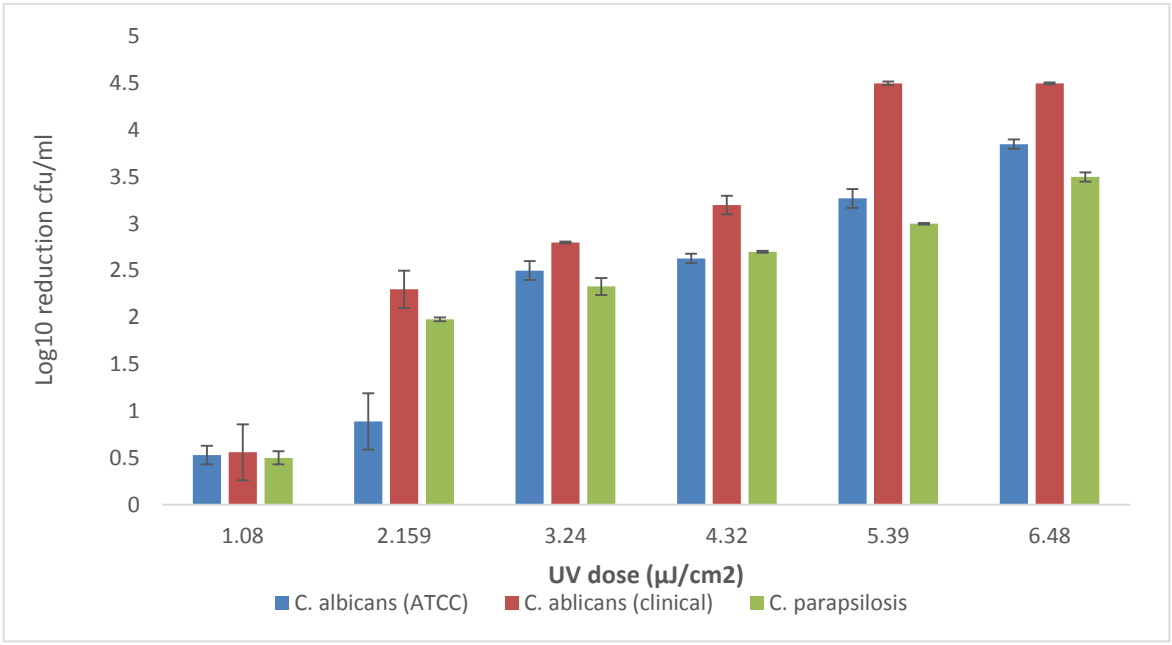


(a)

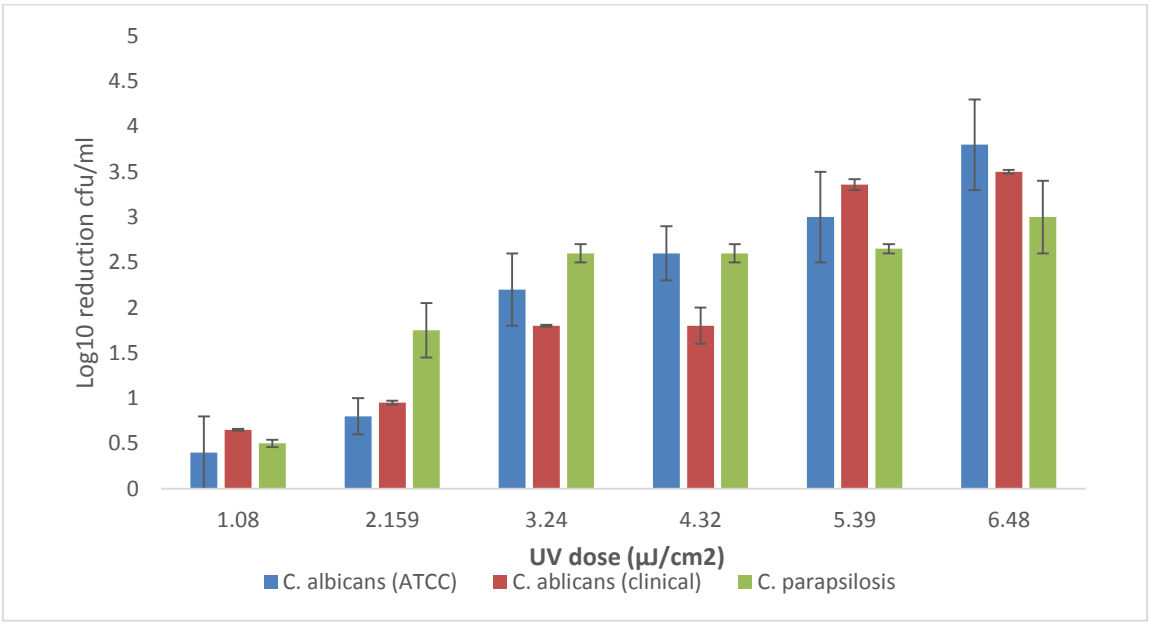


(b)

Figure 3.: Pulsed light inactivation of 48 hour Candida species biofilms grown on (a) PVC and (b) stainless steel surfaces (+/-S.D). n = the mean of 3 separate replicates of individual experiments.



(a)



(b)

Figure 3.: Pulsed light inactivation of 72 hour Candida species biofilms cells grown on (a) PVC and (b) stainless steel surfaces (+/-S.D). **n = the mean of 3 separate replicates of individual experiments.**

The PUV system used in this study repeatedly inactivated *Candida* species biofilms on both PVC and stainless steel surfaces. Significant levels of inactivation were obtained for *C. albicans* (ATCC), *C. albicans* (clinical) and *C. parapsilosis* for 48 (figure 3.8) and 72 hour (figure 3.9) biofilm structures. For both time points ca. 3.5 – 4 $\log_{10}$ cfu/ml inactivation of all test strains was achieved with a fluence of 6.48μJ/cm^2. There was no difference in the inactivation rates of *C. parapsilosis* 48 hour and 72 hour biofilms on PVC surfaces. *C. albicans* biofilms appear more UV sensitive at 48 hours with a significant ($p \leq 0.05$) increase in inactivation obtained for each PUV fluence. *C. albicans* clinical isolate showed similar or a slightly decreased level of inactivation at 48 hours compared to 72 hour biofilm formation. 48 hour biofilms on stainless steel surfaces appear more UV sensitive with an increase in inactivation achieved for *C. albicans* up to a UV fluence of 4.32 μJ/cm^2 and for all treatment fluences for *C. albicans* (clinical) and *C. parapsilosis*. At 48 hour biofilm formation, *C. parapsilosis* appears most resistant to UV pulses with both *C. albicans* strains showing similar levels of sensitivity on PVC surfaces. A similar trend was observed for biofilms grown for 48 hours on stainless steel surfaces. The order of increasing sensitivity to UV fluence was *C. parapsilosis*, *C. albicans* and *C. albicans* (clinical) on PVC surfaces and *C. albicans* (clinical), *C. parapsilosis* and *C. albicans* on stainless surfaces for 72 hour biofilms structures. In general it was found that planktonic cells are more sensitive to PUV than attached cells on either surface material. The order of sensitivity to UV pulses was the same for fungal suspensions and biofilms on stainless steel surfaces, meaning that planktonic cells treated in suspension had the same level of susceptibility as cells in biofilms on stainless steel surfaces to pulsed light. *C. parapsilosis* proved the most sensitive test strain on agar surfaces and PVC attached biofilms.

3. 5. Detection of *Giardia lamblia* in biofilm structures using RT-PCR

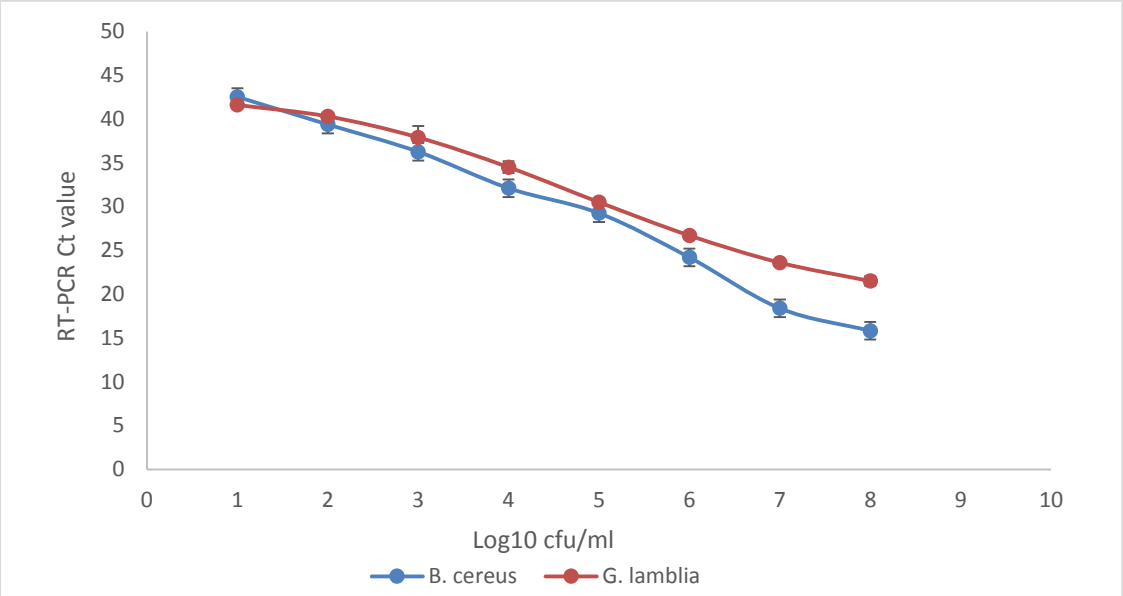


Figure 3.: DNA standard curve as determined by real time PCR analysis for *Bacillus cereus* and the parasite species *Giardia lamblia* (log_{10} cfu/ml) (+/-S.D) using species specific primers.

n = the mean of 3 separate replicates of individual experiments.

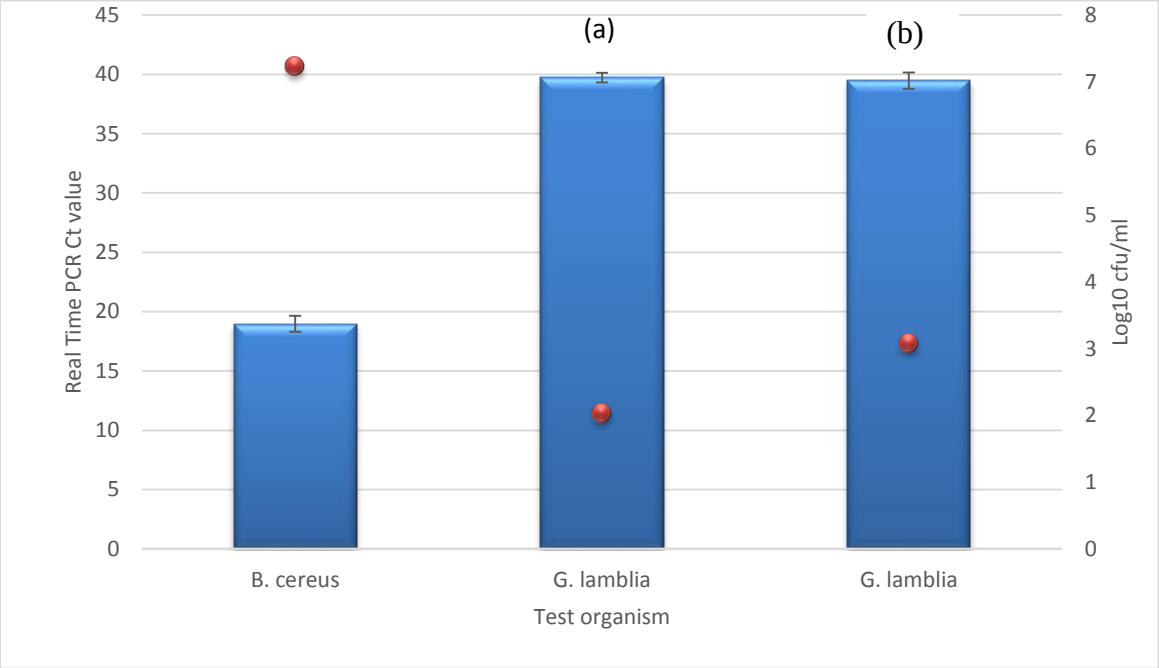


Figure 3.: Real time PCR Ct value for microbial test species *Bacillus cereus* and corresponding cell count in log10 cfu/ml as determined by using the equation of the line of the standard curves for *Giardia lamblia* as detected in *B. cereus* biofilms on (a) PVC and (b) stainless steel surfaces (+/- S.D). n = the mean of 3 separate replicates of individual experiments.

The determination of cell number for *B. cereus* biofilms via PCR was slightly higher than the standard cell count method. A Ct value of 18.9, corresponding to a cell count of ca. 7 $\log_{10}$ cfu/ml for both materials was determined by analysis of the standard curve (figure 3.10). An important fact to note is that PCR detects the presence of target DNA, but cannot differentiate between live and dead cells. In contrast, the standard cell count technique reports viable cell numbers only via the enumeration of colonies grown on nutrient agar. In this case a biofilm viable cell density of 5 $\log_{10}$ was formed, indicating that approximately 2 $\log_{10}$ of non-viable cells were also present in the biofilm matrix as detected by PCR. Studies have shown that with greater incubation times (up to and exceeding 96 hours) no increase in cell number occurred for biofilms populations, suggesting the presence of a stationary phase or steady state of biofilm growth. PCR analyses showed the presence of non-viable cells at 72 hours, further confirming that incubation for 72 hours provided an optimal period of time for biofilm formation, after which cell death occurs to some extent. These findings correspond to that of Senevirantne *et al.*, 2013, who concluded that 72 hours was also the optimal incubation time for the growth of *Enterococcus faecalis* biofilms. Therefore, the findings of this study suggest that 72 hour duration of incubation is sufficient to reproduce a robust, densely populated biofilms of *B. cereus, E. coli, L. monocytogenes* and *S. typhimurium* using a CDC reactor. Consequently, 72 hour biofilms were used for inactivation studies for all test species.

G. lamblia were detected in the *B. cereus* biofilms at a concentration of between 2 and 3 $\log_{10}$ for PVC and stainless steel surfaces by PCR (figure 3.11). Additionally, PCR proved a more efficient reliable method of detecting *Giardia* than the use of specific dyes. Fluorescent dye staining of biofilms containing cysts greatly underestimated the number of organisms present. A maximal cyst count of 14 (+/-4) for *G. lamblia* was determined via fluorescent staining.

4. Discussion

4. 1. Comparative study on the Caco-2 and HCT-8 cell lines as infectivity models for *G. lamblia*.

This study investigated the efficacy of using novel pulsed UV light technology as a disinfection tool for *G. lamblia* and used a combined cell culture method - qPCR for the detection of same. The relevance of this study going forward bears great significance in new effective disinfection technologies that can be applied in clinical veterinary settings. While there is growing interest in the use of in vitro cell culture assays to study the pathogenesis and infectivity of waterborne Cryptosporidium infection post disinfection (Hijjawi, 2010; Garvey *et al*., 2010), there is however, even with the completion of this study, a scarcity of significant information on the detection and efficacy of emerging technologies for disinfection of *G. lamblia*.

Several comparative assays have been used to test infectivity and viability of Giardia cysts, including in vivo mouse infectivity and vital staining techniques (as seen in figure 3.1), the latter proving consistently inadequate at determining UV inactivation of these parasites (Craik *et al.,* 2000). Until recently, based on viability assays as measured exclusively via excystation or vital dye staining (Mofidi *et al*., 2002), it was understood that UV light could not adequately inactivate *G. lamblia*. The importance of developing a Real Time PCR assay for the viability assessment of UV treated parasites is based on excluding the need for an in vivo model allowing for the rapid assessment of disinfection techniques used. As an essential requirement for this study a cell culture based assay needed to be developed to support this novel RT-PCR technique. Considerations in choosing the appropriate cell lines for this study included the need for the cells to correspond to the target area of infectivity of the host, the intestinal tract. Given that *G. lamblia* is a parasite of the intestine, HCT-8 cells were chosen to mimic the small intestine epithelial cells, while Caco-2 cells were chosen as a comparison to HCT-8s as they represent the colon. Similarly, Garvey *et al.,* (2010) developed an *in vitro* cell culture assay for

assessing efficacy *Cryptosporidium parvum* detection that was capable of replacing *in vivo* mice models. By developing such an assay, inconsistencies no longer prove problematic for future studies e.g. age, sex, and health status of the in vivo model as well as in vitro models being ethically unassailable. Until recently when Garvey *et al.*, (2014) commenced work on the parasite *G. lamblia*, no prior study had focused on the efficacy of using new innovative disinfection technologies for the destruction of the pathogenic *G. lamblia*. This could be due to the fact that ethical approval is obligatory when using *in vivo* infectivity rodent models to test the efficacy of the disinfection methods or the high complexity of cell culture requirement for the flagellated protozoan. Studies by Garvey *et al*., 2014 demonstrated that the mechanism of *Cryptosporidium* cellular infectivity differed to that of *Giardia* where the latter was observed to be non-cell membrane invasive but only requiring attachment to epithelial surfaces to initiate and enable infection (Garvey *et al*., 2014). As with this study an increase or decrease in the amount of host cells available for Giardia trophozoites significantly affected its infection and subsequent intracellular proliferation and PCR detection. This is mainly attributed to the surface area available for parasite attachment. The loss of viability and detachment which occurred with the CaCo-2 cells may have led to the decrease in infectivity that was observed for this cell line following exposure to viable *Giardia* trophozoites. Exposure of Caco-2 cells to *Giardia* consistently resulted in apoptotic or early phase cell death in this monolayer, which was not observed with similarly challenged HCT-8 cells (Garvey *et al*., 2014). Previous studies conducted by Cotton *et al.*, (2011) showed that genes associated with apoptosis are up-regulated in cells exposed to *Giardia* and heightened rates of epithelial apoptosis occurs shortly after exposure to *Giardia* trophozoites, which was observed both *in vitro* and *in vivo*. This marked difference in pathogenesis between these two veterinary relevant parasites may be attributed to *Cryptosporidium parvum* inhibiting apoptosis at the trophozoite stage or promoting apoptosis sporozoite and merozoite stages in HCT-8 cells (Panora *et al.,* 2007).

Giardia lamblia predominantly colonises the proximal small intestine and this is the primary site of infection following ingestion of the parasite. Therefore, the HCT-8 cell line which is of ileum origin is an ideal candidate for *in vitro* infectivity studies. The Caco-2 cell line has its origin in the colon and this difference in gastrointestinal location may contribute to the ability of the cells to withstand parasitic infection for both the *Giardia* and *Cryptosporidium* species. Therefore, the findings of this study show that the HCT-8 cell line was a superior host to infectivity than the CaCo2 cells. This is evident by the lower detection of parasitic DNA (figure 3.3) and a more stable cell monolayer and microscopically as detached and apoptotic monolayers. Consequently, the HCT-8 cell line was deemed more suitable as an in vitro host cell type for *Giardia* infectivity and therefore used throughout this study. These findings are comparable to the studies of Hijjawi (2010) who reported that the HCT-8 cells were superior to that of eleven other human cell lines tested when carrying out similar parasite infectivity studies (Hijjawi, 2010). Furthermore, Garvey *et al.,* 2014 demonstrated that HCT-8 cells to be more robust as they continued to proliferate whether the cells were infected or not.

4. 2. Comparative LP- UV and PUV inactivation of veterinary relevant parasite Giardia lamblia.

Table 3.1 and 3.2 detail the inactivation of Giardia test species following exposure to LP and PUV light. Results show that a significant level of parasite inactivation was achieved with both UV systems (Table 3.1). However the pulsed light approach resulted in a higher rate of inactivation with a greatly reduced rate of energy requirement than the LP system, operating at 10^{-3} mJ (μJ) as opposed to mJ. Indeed, with a treatment time of 210 seconds equivalent to LP dose of 113 mJ/cm^2 or PUV dose of 22.68 x10^{-3} mJ/cm^2 a maximal 1.7 $\log_{10}$ and 2.8 $\log_{10}$ inactivation of Giardia was achieved with LP UV and PUV respectively. This demonstrates that even with a greatly reduced energy consumption rate a significantly ($p \leq 0.05$) increased level of inactivation was achieved using the pulsed UV approach. Results also show that with

the LP system a 1.9 $\log_{10}$ reduction was achieved with a dose of 10 mJ/cm^2, this did not increase significantly with a further increase in applied UV dose. Indeed a plateau effect was observed with a dose exceeding 10.8 mJ/cm^2 and up to 49 mJ/cm^2. These findings correspond to that of Craik *et al.,* 2000 and Campbell and Wallis, 2002 where studies show that 99% of *Giardia* inactivation was achieved below 8 mJ/cm^2 and 10 mJ/cm^2 respectively using a medium pressure lamp and *in vivo* infectivity in mice and gerbil models. At doses exceeding 8 mJ/cm^2 and as high as 130 mJ/cm^2 there was no significant increase in the inactivation of *Giardia* cysts (Craik *et al.,* 2000). The similarities in these findings using a cell culture based assay to that of animal infectivity suggest that the former *in vitro* based approach may indeed show levels of infectivity to that of the *in vivo* test system. A similar trend where *C. parvum* showed increased sensitivity to medium pressure UV than *Giardia* was reported by Belosevic *et al.,* 2001.

The pulsed light system provided a ca. 3 $\log_{10}$ (99.9%) inactivation of Giardia with a dose 22.68 x10^{-3} mJ/cm^2. Additionally, the plateau effect observed with the LP system was not evident with every increase in PUV dose a corresponding increase in inactivation was achieved (table 3.1). Previously published work of Garvey *et al.,* 2010 detailing the inactivation of *Cryptosporidium parvum* reported a 3.3 $\log_{10}$ inactivation following a pulsed UV dose of 9.72x10^{-3} mJ/cm^2. This report also showed the correlation between mice infectivity and an *in vitro* HCT-8 CC qPCR assay for the disinfection kinetics of *C. parvum* with pulsed UV light. By comparison *Giardia lamblia* appears more UV resistant than *C. parvum* at doses up to 9.72x10^{-3} mJ/cm^2 with a 1.2 $\log_{10}$ and < 4 $\log_{10}$ inactivation obtained for *Giardia* and *Cryptosporidium* (Garvey *et al.,* 2010) respectively.

Table 3.2 highlights the disparities in the various viability assays currently in use to assess Giardia inactivation. As shown the DAPI/PI dye exclusion and in vitro excystation PCR assay (without cell culture) greatly overestimated the viability of treated cysts compared to the cell

culture PCR assay. This was the irrespective of the treatment system used (LP or PUV). As expected, the dye based assay was not suitable for determining inactivation rates post treatment due to fact that such assays are based solely on membrane damage. It has been well documented that UV disinfection is centred on the principal of genetic damage resulting from the absorbance of photons of UV light. PCR amplification of parasite DNA (without cell culture) also greatly overestimated the extent of Giardia inactivation (table 3.2), due to the fact that PCR alone does not differentiate between live or dead trophozoites. These findings highlight the importance of the additional cell culture step pre DNA extraction, as supplying a host cell for infectivity allows for the parasite reproduction cycle to initiate. By utilising this approach, only viable infective trophozoites were amplified giving a direct and accurate measurement of viability pre and post treatment.

4. 3. PUV inactivation of veterinary relevant microbial test strains.

All Gram positive and Gram negative bacterial test species proved susceptible to PUV light inactivation. A pattern of degree of susceptibly was evident (figure 3.4 a and b), this being that the test species in liquid suspensions were more resistant at equal doses to that of surface treated organisms. In Figure 3.4 at a UV dose of 2µJ/cm^2 a 7 $\log_{10}$ reduction of *E. coli was* achieved on agar surfaces (figure 3.4a) with a 5 $\log_{10}$ reduction seen in liquid suspension (figure 3.4b), a difference of 2 $\log_{10}$ cfu/ml. This pattern falls true also for *B. cereus, L. monocytogenes* with reduction differences at a treatment dose of 6µJ/cm^2, where a difference of 2.5 $\log_{10}$ cfu/ml and 5 $\log_{10}$ cfu/ml respectively was obtained. The exception to this pattern was *S. typhimurium* which proved more resistant when treated on agar surfaces than when treated in liquid suspensions. A UV dose of 6µJ/cm^2 gives a $\log_{10}$ reduction of 4.5cfu/ml on surfaces but a $\log_{10}$ reduction of 9.5cfu/ml in liquid suspension, giving a difference of 5cfu/ml. *E. coli* showed the greatest level of inactivation on agar surfaces with complete inactivation of an initial concentration of ca. 9 $\log_{10}$ with as little as 5 µJ/cm^2 of pulsed light. The order of decreasing

sensitivity for test strains was *E. coli*, *L. monocytogenes*, *B. cereus* and *S. typhimurium* on surfaces. When treated in suspension this sensitivity changed with *L. monocytogenes* showing the highest resistance to PUV treatment and *S. typhimurium* showing the greatest sensitivity to pulsed light for all treatment doses ($p<0.05$). Indeed, a maximal 9 $\log_{10}$ inactivation of *S. typhimurium* was achieved with 5.5 µJ/cm^2 compared to a 2.5 $\log_{10}$ for *L. monocytogenes.* This same dose resulted in a 3 and 5 $\log_{10}$ inactivation of *B. cereus* and *E. coli* respectively, highlighting the significant difference in susceptibility to pulsed UV. These findings correlate with Cheigh *et al*., 2012 where *E. coli* also proved more sensitive to PL than *L. monocytogenes* when treated in suspension.

Candida and *Saccharomyces* test species also proved susceptible to PUV light inactivation (figure 3.5 & 3.6). All yeast strains in liquid suspensions were more resistant to PUV treatment that those on agar surfaces. *C. kruesi* treated at a UV dose of 5µJ/cm^2 resulted in a 7.3 cfu/ml Log_{10} reduction on surface yet only a reduction of 5 Log_{10} cfu/ml in suspension, a difference of 2 $\log_{10}$ cfu/ml is seen. *C. parapsilosis* treated at a UV dose of 5µJ/cm^2 a $\log_{10}$ reduction of up to 7.3 cfu/ml on surface agar yet only a $\log_{10}$ reduction of 4.5cfu/ml, giving a difference of 2.8cfu/ml. *S. cerevisiae* treated with a UV dose of 7µJ/cm^2 showed a reduction of 3.7 Log_{10} cfu/ml, this is similar to the work of Rowan *et al,* 1999 with a 5 log*10* reduction was achieved for *S. cerevisiae* on agar surface in 5 pulses using a UV dose of 0.7J.cm^2 (Rowan *et al*, 1999). When a comparison of treatment dose and microbial test species is looked at in detail (figure 3.4, 3.5 & 3.6), it is evident that bacterial test species are more susceptible to UV light inactivation treatment than yeasts. This corresponds with *in vitro* studies carried out by Rowan *et al*. 1999 and Anderson *et al.* 2000, both having demonstrated that fungi are more resistant to pulsed light than bacteria. This study also mirrors the findings of previous studies by Krishnamurthy *et al*., 2007 and Marquenie *et al*. 2003 as it was found that pulsed UV treatment is more effective on solid surfaces than in liquids.

4. 4. Growth and sterilisation of microbial & fungal biofilms on surfaces present in the veterinary environment.

Biofilm communities are the natural state of microbial habitat where they are found attached to biotic or abiotic surfaces more so than planktonic free floating cells. The change from a planktonic free floating cell to that of a biofilm sessile cell induces physiological changes in bacteria. This occurs via a series of gene expression alterations which include gene repression and induction. It is the induction of genes, relating to antibiotic resistance that leads to the increased pathogenicity of sessile bacteria over their planktonic counterparts. Additionally, the adaption to this new environment and associated living conditions induces genes that encode for new important proteins and enzymes. Quorum sensing and response mechanisms within biofilms allows for gene regulation and plays an important role in pathogenicity and virulence of organisms (Clusterbuck *et al.,* 2007). Cell adhesion is believed to trigger gene expression responsible for the control of microbial products necessary for adhesion to a surface (Costerton and Lappin-Scott., 1995). The enzyme polyphosphate kinase (PPK) is one such protein, linked to the thickness of biofilms (Clusterbuck *et al.,* 2007) adding to the EPS matrix and is believed to subsequently limit antibiotic penetration of a biofilm. Consequently, this causes an increased resistance to antibiotics and disinfectants commonly observed with these complex structures (Aguilar-Romero *et al.,* 2010). Regardless of this, planktonic culture remains the main mechanism for microbiological studies such as disinfection (Otter *et al.,* 2014). Furthermore, the sensitivity of planktonic cells to disinfection has been used as an indication of biofilm sensitivity and resistance (Buckingham-Meyer *et al.,* 2007). However, disinfection studies based on actual biofilm communities is much more representative of the environmental situation. The findings of this study show that the use of the CDC biofilm reactor allows for the growth of biofilms with high cell densities attached to surfaces in a reproducible manner. Such methods ensure that the biofilm structures possess the key characteristics of real life

biofilms for the test species in question. This study assessed the sensitivity of microbial biofilm structures to pulsed light treatment and provides a direct relationship between treatment fluence and loss in sessile cell viability. Consequently an important aspect of clinical disinfection and disease prevention is dealt with.

Biofilms are often associated with a number of persistent infections that poorly respond to antibiotics (Hall-Stoodley, 2004) and can be the cause of detrimental losses within the agricultural and veterinary environment. In 2013 for example, New Zealand's leading export market to China was temporarily closed due to a botulism scare in whey products originating from Fonterra Cooperative Group. 95% of all dairy products produced in New Zealand are exported, feeding $NZ11bn directly into the New Zealand economy. Unsterilized pipes were the source of the contamination on that particular occasion.

The formation of biofilms on surfaces and in other inaccessible areas, often harbouring pathogenic bacteria, fungi, viruses or parasites, raises concerns to the health of inpatients and the public. Therefore, the inactivation of these resilient bacterial communities is important in order to reduce the risk to veterinary staff and in patients contracting a nosocomial disease while hospitalised and at work. Furthermore, Neely and Orloff, 2001 have shown that fungal pathogens also have the ability to survive on clinical surfaces. Established biofilms on these surfaces pose a difficult challenge to hospital cleaning and disinfection, due to their resistance to biocides and difficulty to remove by detergent cleaning (Otter *et al.*, 2014). Fungal infections are increasing at a disturbing rate affecting a growing population of severely ill patients which poses important challenges for the health care professionals. Studies by Chandra *et al.*, 2001 have shown that cellular resistance to biocides increased as the biofilm structure matured, with a 72 hour biofilm of *C. albicans* showing a dramatically increased level of resistance than their earlier stage biofilm counterparts (Chandra *et al.*, 2001). A means of inactivating planktonic and sessile cells which renders the surface free of pathogenic species is essential to prevent

patient infection or the contamination of medical materials. Research by Garvey *et al*., 2013 concluded that the CDC reactor allows for the formation of more densely populated bacterial biofilms than alternative methods such as microtiter plates (Nailis *et al.,* 2010) and attributed this to the availability of nutrients within the reactor. Studies assessing *in vivo* models for catheter associated *Candida* infections such as microscopic structure analysis indicate that the *in vitro* techniques such as the CDC reactor show similarly structured biofilm communities than those found *in vivo* (Lopez-Ribot, 2005). Furthermore, *in vitro* studies have shown that *Candida* can survive in the low iron environment found in the tissues surrounding implanted devices such as catheters, with the additional factor of its filamentous life cycle making it proficient at colonising inert surfaces such as PVC (Suci and Tyler, 2002). These studies suggest that *in vitro* model systems mimic *in vivo* events indicating that the research outputs made are clinically relevant. For PUV inactivation studies both the stainless steel and PVC coupons were exposed to varying UV doses on both sides to ensure complete biofilm exposure. Replicate results indicate that this pulsed UV system was effective at inactivating all test species at 8 μJ per pulse. Indeed substantial amounts of inactivation were achieved with as a little as 1 $\mu J/cm^2$ for bacterial biofilms on PVC and SS surfaces, (figure 3.7 a and b). Additionally, for all strains a consistent pattern emerged where for each treatment dose resulted in a significant difference in inactivation rates. Furthermore, the PUV system tested repeatedly inactivated *Candida* species biofilms on both PVC and stainless steel surfaces. Significant levels of inactivation were obtained for *C. albicans* (ATCC), *C. albicans* (clinical) and *C. parapsilosis* for 48 (figure 3.8) and 72 hour (figure 3.9) biofilm structures. For both time points ca. 3.5 – 4 $\log_{10}$ cfu/ml inactivation of all test strains was achieved with a fluence of 6.68 $\mu J/cm^2$. There was no difference in the inactivation rates of *C. parapsilosis* 48 hour and 72 hour biofilms on PVC surfaces. *C. albicans* biofilms appear more UV sensitive at 48 hours with a significant ($p \leq 0.05$) increase in inactivation obtained for each PUV fluence. *C. albicans*

clinical isolate showed similar or a slightly decreased level of inactivation at 48 hours compared to 72 hour biofilm formation. 48 hour biofilms on stainless steel surfaces appear more UV sensitive with an increase in inactivation achieved for *C. albicans* up to a UV fluence of 4.32 μJ/cm^2 and for all treatment fluences for *C. albicans* (clinical) and *C. parapsilosis*. At 48 hour biofilm formation, *C. parapsilosis* appears most resistant to UV pulses with both *C. albicans* strains showing similar levels of sensitivity on PVC surfaces. A similar trend was observed for biofilms grown for 48 hours on stainless steel surfaces. The order of increasing sensitivity to UV fluence was *C. parapsilosis*, *C. albicans* and *C. albicans* (clinical) on PVC surfaces and *C. albicans* (clinical), *C. parapsilosis* and *C. albicans* on stainless surfaces for 72 hour biofilms structures. The order of sensitivity to UV pulses was the same for fungal suspensions and biofilms on stainless steel surfaces, meaning that planktonic cells treated in suspension had the same level of susceptibility as cells in biofilms on stainless steel surfaces to pulsed light. *C. parapsilosis* proved the most sensitive test strain on agar surfaces and PVC attached biofilms.

In general it was found that planktonic cells are more sensitive to PUV than attached biofilm cells on either surface material.

Further studies are warranted on the photo reactivation ability of organisms within the biofilm structure following PUV treatment. However, it is worth noting that previously published studies by McDonald and Curry, 2001 postulate that the high proton flux from a pulsed light source overwhelms the cellular repair mechanisms of treated organisms.

4. 5. Studies to determine the entrapment of parasite species within these biofilm structures

Biofilms structures of a bacterial and/or fungal nature when attached to surfaces can entrap organisms such as viruses or parasites within their matrix, providing a protective environment. This harbouring of parasites such as Giardia means that they are shielded somewhat from the action of various disinfection processes. This represents a serious threat to the veterinary industry in relation to the contraction of nosocomial infections. Detachment from biofilms can occur by continuous erosion from both physical cleaning and chemical disinfection (Wingender and Flemming., 2011). The studies described herein show that Giardia parasites were indeed trapped in *Bacillus cereus* biofilms on both surface types (figure 3.11).

Findings indicate that PCR proved a more efficient reliable method of detecting Giardia with biofilm structures than the use of specific dyes. Fluorescent dye staining of biofilms containing cysts greatly underestimated the number of organisms present. Issues arose in relation to non-specific binding of dyes to biofilm constitutes believed to be EPS components resulting in unreliable counting of parasite numbers. These findings confirm that Giardia can quickly attach or become entrapped in bacterial biofilms. The detection of these pathogens within biofilm structures has important public health implications in relation to animal and human exposure. The robust, disinfection resistant nature of biofilms and these parasites themselves increases the probability that the survival and detachment of biofilm-associated viable parasites may occur at concentrations exceeding that required for infection. This possibility needs to be considered in risk assessments relating to the cleaning of veterinary environments particularly where animals are housed and fed.

4. 6. Conclusions

- UV light would be an effective sterilization technique in a veterinary clinical setting once regular cleaning had taken place.
- Findings indicated that current *Giardia* detection methods are limited to using vital stains before and after cyst excystation are not appropriate for monitoring or evaluating cyst destruction post PUV-treatments.
- Use of the human ileocecal HCT-8 cell line was superior to that of the human colon Caco-2 cell line for *in vitro* culture and determining PUV sensitivity of treated cysts.
- The use of this HCT-8 cell culture assay may replace use of animal models for determining disinfection performances of PUV for treating *G. lamblia*
- The extraction and amplification of the parasitic DNA via real time PCR provides a rapid measurement of infective parasite numbers allowing for the measurement of live or dead parasites post PUV treatment.
- Findings demonstrate the use of pulsed UV light to be effective in the inactivation of clinically relevant microbial species on surfaces commonly associated within the clinical veterinary environment.
- Based on findings from this study, and in an attempt to be proactive in the reduction of biofilm adhesion, Stainless Steel should be chosen instead of PVC material when choosing kennels, feed bowls etc for use in a veterinary clinic.

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APPENDIX I

Pulsed light disinfection of veterinary relevant microbial biofilms harboring parasite species *Cryptosporidium parvum* and *Giardia duodenalis* as detected via real time PCR

Mary Garvey[1]*, Gillian Coughlan[2, 3], Noel Murphy[2] and Neil Rowan[3]

[1]Department of Life Sciences, Institute of Technology Sligo, Sligo, Ireland

[2]Department of Biology, National University of Ireland Maynooth, Maynooth, Ireland

[3]Bioscience Research Institute, Athlone Institute of Technology, Athlone, Ireland

Running title: Pulsed light for the disinfection of veterinary biofilms

*Corresponding author: Dr Mary Garvey

Department of Life Sciences

Institute of Technology Sligo

Ash Lane, Sligo

Ireland

garvey.mary@itsligo.ie

Phone: +353719305841

Abstract

The presence of pathogenic organisms namely parasite species and bacterial biofilms in veterinary settings is a public health concern in relation to human and animal exposure. Veterinary clinics represent a significant risk factor for the transfer of pathogens from housed animals to humans especially in cases of wound infection and the shedding of faecal matter. This study aims to provide a means of detecting veterinary relevant parasite species in bacterial biofilms, and to provide a means of disinfecting these biofilms. A real time PCR assay was utilized to detect parasite DNA in *Bacillus cereus* biofilms on stainless steel and PVC surfaces. Results show that both *Cryptosporidium* and *Giardia* attach to biofilms in large numbers (100-1000 oo/cysts) in as little as 72 hours. Pulsed light successfully inactivated all test species (*Listeria, Salmonella, Bacillus, Escherichia*) in planktonic and biofilm form with an increase in inactivation for every increase in UV dose.

Keywords: biofilms, veterinary, *Giardia*, *Cryptosporidium,* PCR, pulsed light,

Introduction

The prevention and control of veterinary related infections is an important aspect of public health and safety due to the occurrence of zoonotic infections. The spread of pathogenic species within veterinary practices can lead to infection of both the housed animals and veterinary staff. Veterinary clinics are a connection of human and animal interaction, often in situations dealing with infected wounds or faecal matter. This is a significant concern for immunocompromised individuals who are animal owners. Animal associated pathogens of concern to immunocompromised persons include *Cryptosporidium, Salmonella, Listeria, Bacillus, Escherichia coli, Campylobacter* and *Giardia* (Grant and Olsen, 1999). Furthermore, many research studies have highlighted the connection between the spread of pathogenic organisms from surfaces to patients (Gebel *et al.,* 2013). Consequently, the use of surface disinfectants for the control of pathogens in clinical and veterinary settings has become important due to the increase in antibiotic resistant microbial species and zoonotic infections. However, issues have arisen where some pathogens have shown resistance to commonly used chemical based disinfectants. Such pathogens include the parasite species *Cryptosporidium* and *Giardia*, bacterial endospores and bacterial biofilm structures. Planktonic microbial cells are able to attach to and colonise environmental surfaces by producing an extracellular polymeric substance (EPS), these adherent (sessile) cells are referred to as biofilms. The descriptive terms sessile and planktonic are used to describe surface adherent and free floating bacterial cells respectively. Veterinary important species such as *Listeria, Escherichia, Bacillus* and *Salmonella* are capable of producing these biofilm structures allowing them to gain resistance to standard chemical disinfection methods. Indeed, biofilms or sessile communities are believed to be the causative agent in diseases such as pneumonia, liver abscesses, enteritis, wound infections and mastitis infections in animals (Clutterbuck *et al.*, 2007). In addition, in hosts with functioning innate and adaptive immune responses, biofilm-based infections are

often very persistent and remain unresolved. In fact surrounding tissues often undergo extensive damage by immune complexes and invading neutrophils when trying to eradicate the infection (Stewart and Costerton, 2001).

The prevention of biofilm formation would provide the best control measures for these robust structures; however, there is no agent available that will prevent cell adhesion and biofilm formation. Current methods rely on the use of disinfection agents and regular cleaning of surfaces exposed to possible pathogens. Research has indicated that sessile communities can be up to 1000 times more resistant to chemotherapeutics such as chlorhexidine than their planktonic counterparts. Furthermore, resistant bacteria originated in sessile communities can spread from animal to animal through veterinary staff, veterinary surfaces and equipment or farm equipment such as feeders and water dispensers (Aguilar-Romero *et al.,* 2010) resulting in extended infection problems. Biofilm structures are also capable of trapping or incorporating other pathogenic species including viruses and parasites such as *Giardia* and *Cryptosporidium* (DiCesare *et al.,* 2012). Harbouring of such species shields them from cleaning and disinfection techniques, increasing their already high resistance to such treatments. Studies have shown that biofilms represent a significant, long-term reservoir for pathogens such as *Cryptosporidium* and *Giardia* which can be released back into water. Thus, explaining the presence of parasites in water networks long after disinfection protocols are completed following an outbreak. Indeed, the continued presence of *Cryptosporidium* in a drinking water system following an outbreak in England was attributed to the presence of biofilm structures on the piping network (Wingender and Flemming, 2011). Such findings indicate that alternative ways of pathogen inactivation in the veterinary setting must be provided. Ultraviolet (UV) light is well known for its antimicrobial activity, due to its bacteriostatic properties preventing bacterial cell replication. Additionally, research focusing on the use of a pulsed light system for the inactivation of parasite species and bacterial endospores has shown this system to be highly

efficient (Garvey *et al.*, 2014). Pulsed light technologies differ from standard UV lamps in their mode of delivery, penetration depth and wavelength range (Garvey *et al.*, 2014) making them a more potent disinfection system. Here we report on the use of a pulsed light system for the disinfection of veterinary relevant biofilms on polyvinylchloride (PVC) and stainless steel surfaces. The use of polymerase chain reaction (PCR) methods provides a rapid species specific means of identifying species type and cell numbers present. Indeed, PCR methods have been used extensively to detect and quantify bacterial cells in food products and in biofilms (Pan and Breidt, 2007). Therefore, the present study also utilised a real time PCR assay to determine the extent at which *Bacillus* biofilm structures incorporated parasite species into their matrix, subsequently providing shelter from disinfection techniques.

Methods

Pulsed UV light

The PUV machine used throughout this study was sourced through Samtech Ltd, Strathclyde, Scotland, UK. The bacteriostatic effects of pulsed light are caused by the rich and broad-spectrum UV content, the short duration, and the high peak power of the pulse. The system was used as per Garvey *et al.*, (2014) and Garvey *et al.*, (2010) and is therefore not described in further detail herein.

Microbial test species

For this study a range of veterinary relevant microbial species *Listeria monocytogenes* (ATCC 11994), *Bacillus cereus* (ATCC 11778), *Salmonella typhimurium* (ATCC 13311) and *Escherichia coli* (ATCC 11775) were chosen for biofilm formation and pulsed light inactivation studies. All strains were cultured and maintained nutrient agar and nutrient broth (Cruinn Diagnostics Ltd, Ireland) at 37^{o}C. *Giardia duodenalis* cysts and *Cryptosporidium parvum* oocysts were purchased from Waterborne Inc USA. Oocysts and cysts 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 at 4°C. Species identity was confirmed by a dye staining method comprising of propidium iodide (PI) 1 mg/ml in 0.1 M sterile PBS and 4', 6'-Diamidino-2-Phenylindole (DAPI) 2 mg/ml in methanol and a fluorescein-labelled mouse-derived monoclonal antibody Giardi-a-Glo™ or Crypt-a-Glo™ (Waterborne Inc, New Orleans, USA) Oo/cysts were counted using a haemocytometer and inverted microscope (Olympus, CKX41) with camera (Olympus, IX2-SLP) attached.

Growth of sessile communities using Centers for Disease Control (CDC) biofilm reactor

The CDC biofilm reactor (Biosurface Technologies Corp, Bozeman, Montana, USA) was used for the growth of biofilm structures as per the recommended procedure of the American Society for Testing and Materials (ASTM). Furthermore, the CDC reactor is a recognised method for the growth of biofilms under high shear and continuous flow (Coenye and Nelis, 2010) and is of sufficient capacity to provide numerous samples of biofilms for disinfection studies. For this study both PVC and stainless steel coupons were chosen as biofilm growth surfaces as both materials are commonly used in veterinary settings and are excellent matrixes for biofilm adhesion and proliferation.

For the growth of microbial biofilms methods were followed as per the recommended procedure for continuous fluid shear flow biofilm formation (ASTM E2562-12 2012) and Garvey *et al.,* 2014. The reactor was prepared containing 350 mL of tryptone soya broth (TSB) and 2% glucose; ensuring disks were completely submerged and autoclaved. 1 mL of a 12 hour microbial culture was added to the reactor chamber to ensure that cells were in the log phase of reproduction. For each test strain the reactor was incubated at 37°C for 72 and 96 hours under rotatory conditions at 125 rpm. To allow for the enumeration of colony forming units (cfu) per microbial biofilm, all coupons were removed aseptically from each reactor rod and

rinsed with sterile phosphate-buffered saline (PBS) to remove any planktonic cells. Biofilms were removed from each coupon by scraping the coupon using a sterile cell scraper into 10 mL of sterile PBS. The standard plate count technique was used to determine the cfu/ml bacterial population in the biofilm as per the recommended procedure (ASTM E2562-12 2012). To allow for the entrapment of parasite test species within the biofilm matrix $1x10^6$ oo/cysts per ml was added to the reactor chamber and incubated for 72 hours. For biofilms containing parasite test species, 1 ml from the 10 ml PBS containing the scrapped biofilm was stained with parasite specific dyes as per previously described to confirm identity and numbers present.

Pulsed light inactivation of planktonic microbial species

*E. coli, S. t*yphimurium, *L. monocytogenes* and *B. cereus* cultures were grown and maintained as previously described. For PUV studies a single colony of the test strain was aseptically transferred to 100 ml of sterile nutrient broth followed by incubation at 37ºC for 12 hours at 125 rpm. For surface treatment 100 µl of an appropriate dilution was spread onto agar surfaces. Test plates were then exposed to pulses of UV light at 16.2J at varying doses at a rate of 1 pulse per second as per Garvey *et al.,* (2014). PUV studies were also conducted on samples diluted from the 12 hour broth in 20 ml final volumes of sterile PBS at 8 cm from the light source, after which 100 µl of treated liquid was transferred to suitable agar and incubated at 37ºC for 24 hours.

Pulsed light inactivation of sessile communities

Coupons were aseptically removed from the reactor, rinsed with sterile PBS and transferred to a sterile petri dish. Samples were exposed to pulses of UV light at 16.2J at 8 cm from the light source at varying UV doses which were obtained by increasing the pulse number. Once treated, coupons were submerged in 10 ml of sterile PBS and surface scraped using a sterile cell scraper to remove the treated biofilms and to allow for the determination of inactivated rates. The liquid was then transferred to a sterile 20 ml container and centrifuged at 800 g for 10 mins to pellet

the cells. The sample was then re-suspended and agitated to ensure biofilm dispersion. Serial dilutions were made from the biofilms suspension and 100 µl spread on triplicate agar plates to determine the cfu/ml of treated samples. This process was repeated for coupons at varying UV doses to determine the Log_{10} reduction obtained with increasing UV dose.

DNA extraction from biofilm structures

Scrapped coupons suspended in 10 ml volumes were centrifuged at 800g for 10 minutes to pellet the cells, followed by re-suspension in 200 µl of sterile PCR grade water. Target DNA extraction was conducted as per kit instructions for *B. cereus* biofilm suspensions using a Roche DNA extraction kit and HP PCR template preparation kit (Roche Diagnostics, Roche, Ireland). All steps were performed as per manufactures instructions with treated and untreated microbial pellets which were suspended in 200 µl of sterile PBS.

Real Time PCR

All primers and probes were sourced from Tib Molbiol, Berlin, Germany. For *B. cereus* the forward primer ACACACGTGCTACAATGGATG and reverse primer AGTTGCAGCCTACAATCCGAA with the taqman probe sequence F-ACAAAGGGCTGCAAGACCGCG—Q coding for the phaC gene was used as per Nayak *et al.,* 2013. Primers coding for β-giardin of *G. duodenalis* were used as per method of Bertrand *et al.,* 2009. The Taqman probe with the following sequence: 5'-FAM TCACCCAGACGATGGA CAAGCCCTAMRA-3 was utilised for this study. For *Cryptosporidium parvum* the 18Si reverse primer 5'- CCTgCTTTAAgCACTTAATTTTC and 18Si forward primer 5'- ATggACAAgAAATAACAATACAgg were utilised. The Taqman probe had the following sequence: 5-'-(6-FAM) ACCAGACTTGCCCTCC (TAMRA). Amplification reactions (20 µL) contained 5 µL of sample DNA (0.5 µM of each primer, 0.2 µM of probe) and 15 µL of reaction buffer (Roche Diagnostic, West Sussex, England). Both positive and negative controls were included in RT-PCR to validate the results. DNase–RNase

free water was used as negative control throughout. Cycling parameters were initial denaturation for 10 min at 95°C followed by 65 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 Nanocycler® device (Roche Diagnostics). Large numbers of cycles were used to ensure detection of low levels of infection. On completion of each RT-PCR run amplification curves were analysed by Nanocycler software (Roche Diagnostics) and a standard curve (figure 1) of cell DNA concentration determined. DNA standards were prepared from fresh cells or oo/cysts ranging in concentration from 10 to 10^8 oocysts or cysts/ml by dilution in PBS following standard viable count determinations.

Statistics

All experimental data is an average of 3 experimental replicates with 3 internal replicates. Bacterial inactivation is expressed as $\log_{10}$ reduction of the untreated control. Student's t-tests and ANOVA one-way model (MINITAB software release 16; Mintab Inc., State College, PA) were used to compare the relationship between UV treatments and bacterial inactivation at 95% level of confidence. Student t-tests were used to determine the relationship between the sensitivity of biofilms from different strains to PL treatment.

Results and Discussion

Sessile communities and parasite detection

The change from a planktonic free floating cell to that of a biofilm sessile cell induces physiological changes in bacteria. This occurs via a series of gene expression alterations which include gene repression and induction. It is the induction of genes, relating to antibiotic resistance that leads to the increased pathogenicity of sessile bacteria over their planktonic counterparts. Additionally, the adaption to this new environment and associated living conditions induces genes that encode for new important proteins and enzymes. Consequently, this causes an increased resistance to antibiotics and disinfectants commonly observed with

these complex structures (Aguilar-Romero *et al.*, 2010). Traditionally, the sensitivity of planktonic cells to disinfection has been used as an indication of biofilm sensitivity and resistance (Buckingham-Meyer *et al.*, 2007). However, disinfection studies based on actual biofilm communities is much more representative of the environmental situation. The findings of this study show that the use of the CDC biofilm reactor allows for the growth of biofilms with high cell densities attached to surfaces in a reproducible manner. Such methods ensure that the biofilm structures possess the key characteristics of real life biofilms for the test species in question. All bacterial strains under study formed densely populated sessile communities on both PVC and stainless steel surfaces after 72 hours. Findings also demonstrate (data not shown) that with longer incubation times, exceeding 72 hours (96 hours), there was no increase in cell number of the biofilms as detected by plate counts. Following 72 hours, a ca. 5 and 6.6 $\log_{10}$ biofilm formed for *B. cereus* and *S. typhimurium* respectively, and a 6 $\log_{10}$ for *E. coli* and 6.5 $\log_{10}$ for *L. monocytogenes* on PVC surfaces. A similar level of cell density was detected on stainless steel surfaces, where a ca. 5 $\log_{10}$ to 6.6 $\log_{10}$ biofilm formed for *B. cereus, L. monocytogenes, S. typhimurium* and *E. coli*. The determination of cell number for *B. cereus* biofilms via PCR was slightly higher than the standard cell count method. A Ct value of 18.9, corresponding to a cell count of ca. 7 $\log_{10}$ cfu/ml for both materials was determined by analysis of the standard curve. An important fact to note is that PCR detects the presence of target DNA, but cannot differentiate between live and dead cells. In contrast, the standard cell count technique reports viable cell numbers only via the enumeration of colonies grown on nutrient agar. In this case a biofilm viable cell density of 5 $\log_{10}$ was formed, indicating that approximately 2 $\log_{10}$ of non-viable cells were also present in the biofilm matrix as detected by PCR. Studies have shown that with greater incubation times (up to and exceeding 96 hours) no increase in cell number occurred for biofilms populations, suggesting the presence of a stationary phase or steady state of biofilm growth. PCR analyses showed the presence of non-

viable cells at 72 hours, further confirming that incubation for 72 hours provided an optimal period of time for biofilm formation, after which cell death occurs to some extent. These findings correspond to that of Senevirantne *et al.,* 2013, who concluded that 72 hours was also the optimal incubation time for the growth of *Enterococcus faecalis* biofilms. Therefore, the findings of this study suggest that 72 hour duration of incubation is sufficient to reproducibly produce a robust, densely populated biofilm of *B. cereus, E. coli, L. monocytogenes* and *S. typhimurium* using a CDC reactor. Consequently, 72 hour biofilms were used for inactivation studies for all test species.

Both parasites species were detected in the *B. cereus* biofilms at a concentration of between 2 and 3 $\log_{10}$ for PVC and stainless steel surfaces by PCR (figure 2). Additionally, PCR proved a more efficient reliable method of detecting *Cryptosporidium* and *Giardia* than the use of specific dyes. Fluorescent dye staining of biofilms containing oo/cysts greatly underestimated the number of organisms present. A maximal oo/cyst count of 10 (+/-2) was measured for *C. parvum* and 14 for *G. duodenalis* (+/-4) via fluorescent staining. Issues arose in relation to non-specific binding of dyes to biofilm constitutes believed to be EPS components resulting in unreliable counting of parasite numbers. These findings confirm that both parasite species can quickly attach or become entrapped in bacterial biofilms. The detection of these pathogens within biofilm structures has important public health implications in relation to animal and human exposure. The infectious dose for *Cryptosporidium* has been established to be less than 20 oocysts (Zambriski *et al.*, 2013) with prolonged infection occurring with little success following medical intervention. The robust, disinfection resistant nature of biofilms and these parasites themselves increases the probability that the survival and detachment of biofilm-associated viable parasites may occur at concentrations exceeding that required for infection. This possibility needs to be considered in risk assessments relating to the cleaning of veterinary environments particularly where animals are housed.

Pulsed light inactivation of planktonic and sessile test species

The impact of pulsed light on microbial species was assessed for surface treated organisms, organisms in suspension and sessile communities. All test strains proved susceptible to the pulsed light treatment, albeit with varying levels of sensitivity as shown in figure 3 and 4. *E. coli* showed the greatest level of inactivation on agar surfaces (figure 3a) with complete inactivation of an initial concentration of ca. 9 $\log_{10}$ with as little as 5 $\mu J/cm^2$ of pulsed light. The order of decreasing sensitivity for test strains was *E. coli, L. monocytogenes, B. cereus* and *S. typhimurium* on surfaces. When treated in suspension this sensitivity changed with *L. monocytogenes* showing the highest resistance to PL treatment and *S. typhimurium* showing the greatest sensitivity to pulsed light (figure 3b) for all treatment doses ($p \leq 0.05$). Indeed a maximal 9 $\log_{10}$ inactivation of *S. typhimurium* was achieved with 5.5 $\mu J/cm^2$ compared to a 2.5 $\log_{10}$ for *L. monocytogenes*. This same dose resulted in a 3 and 5 $\log_{10}$ inactivation of *B. cereus* and *E. coli* respectively, highlighting the significant difference in susceptibility to pulsed light. These findings are in conjunction with Cheigh *et al.*, 2012 where *E. coli* also proved more sensitive to PL than *L. monocytogenes* when treated in suspension. High levels of biofilm inactivation were also achieved for all test strains present on both surface materials (figure 4). For the Gram negative species *E. coli* and *S. typhimurium* a 4.2 and 5.1 $\log_{10}$ reduction in viable cell counts was obtained on PVC surfaces with 5.39 $\mu J/cm^2$ (figure 4a). This same dose resulted in a significantly ($p \leq 0.05$) greater level of inactivation of the same species on stainless steel surfaces, with a maximal 4.2 and 6.6 $\log_{10}$ reduction obtained for *E. coli* and *S. typhimurium* respectively (figure 4b). Both Gram positive species tested showed increased sensitivity on stainless steel surfaces compared to PVC. A dose of 5.39 $\mu J/cm^2$ resulted in a 3.2 and a 4.3 $\log_{10}$ inactivation on PVC and 5.9 and 4.6 $\log_{10}$ inactivation on stainless steel for *B. cereus* and *L. monocytogenes* respectively. A PL dose of 7.38 $\mu J/cm^2$ resulted in complete inactivation of *L. monocytogenes* and *S. typhimurium* of ca. 6.5 $\log_{10}$.

Previous studies by this research group reported a ca. 5 $\log_{10}$ inactivation of *Cryptosporidium parvum* (Garvey *et al.*, 2012) and ca. 1 $\log_{10}$ inactivation of *Giardia duodenalis* (Garvey *et al.*, 2014) with a PL dose of 7.38 μJ/cm^2. Nevertheless, further studies are warranted to determine the exact dose required to inactivate parasites within biofilm matrixes, which will undoubtedly shield parasites to some extent. However, issues are expected to arise in relation to viability determination post treatment and cell culture infectivity. Specifically, issues relating to the sterility of the parasites following extraction from biofilms and subsequent exposure to mammalian cell lines. Nonetheless, PL shows potential for use as a disinfectant for veterinary environments given its highly effective bacteriostatic properties towards bacterial biofilms and parasite species. Regardless of microbial exposure to PL in suspension or on surfaces findings demonstrate that cell inactivation increased significantly ($p \leq 0.05$) with increasing UV dose or treatment time.

In conclusion, the findings reported here contribute to existing literature in many ways.

- Firstly, all veterinary relevant strains produced densely populated biofilms structures on both surface materials used.
- Secondly, PL repeatedly inactivated the range of test species on surfaces and in suspension. Additionally, it provided high levels of biofilm inactivation on PVC and stainless steel surfaces.
- Thirdly, a real time PCR assay proved successful for determining the level of *C. parvum* and *G. duodenalis* present in the biofilms of *B. cereus* where fluorescent staining greatly underestimated the numbers present.
- Finally, pulsed light doses (7.38 μJ/cm^2) which have been previously shown to inactivate both parasite species (*Cryptosporidium* and *Giardia),* have also provided complete inactivation of all biofilms tested.

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