AN ABSTRACT OF THE THESIS OF

Christine C. Nguyen for the degree of Master of Science in Environmental Sciences presented on July 9, 2019.

Title: Ultraviolet Photolytic Inactivation of *Cryptosporidium parvum*, *Bacteriophage MS2* and *Escherichia coli* within a Micro-Reactor

Abstract approved:

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Tala Navab-Daneshmand

Lack of access to safe water is the leading cause of mortality in the world; approximately 3.4 million people die every year from waterborne pathogens. Inadequate access to safe drinking water and sanitation has contributed to an annual average of 1,980,000 deaths of children under five from diarrheal disease. Three of the most common classes of microbial pathogens that confer waterborne illness are bacteria, protozoa and viruses. The World Health Organization international scheme for household water treatment technologies has set standards for a high pathogen removal qualification. For protozoans, virus, and bacteria, 2-log, 3-log and 2-log reductions are required, respectively.

In our lab at Oregon State University, a microreactor device was designed with a novel approach towards pathogen inactivation in drinking water by utilizing ultraviolet degradation. Through its design approach the microreactor maintains a constant flux of photons into the influent and is modular, energy efficient and portable. In this study, we evaluated the efficacy of microfluidic reactor inactivation of fecal indicators from the three main waterborne pathogens: *Escherichia coli*

(bacteria), *Cryptosporidium parvum* (protozoa), and *Bacteriophage MS2* (viruses). The microbes were exposed to ultraviolet light at a variety of residence times within the microreactor and evaluated post treatment for log-removal. *Escherichia coli* was exposed to residence times of: 1.5, 5, 8, 15, and 30 seconds with corresponding average log-removal values of 2.18, 4.57, 7.48, 7.30, and 7.90 CFU/mL post treatment. *Cryptosporidium parvum* was exposed to residence times of 1.57, 15, 120, and 360 seconds and yielded average log removal values of 0.17, 0.95, 1.33 and 1.38 oocyst/mL post treatment. *Bacteriophage MS2* was tested at 1.57, 15, 30, 120, 360, and 480 seconds with average log removals at 0.37, 0.95, 2.78, 3.81, 5.02, 6.96 PFU/mL. We conclude that this microreactor is an effective novel device that meets WHO drinking water regulations within 8 minutes of treatment. The generation of this data set is important in order to develop an investigative tool to create a computational model for the inactivation of microbial pathogens within this microreactor and determine kinetic rate constants for optimization in different applications. This research is also a proof-of-concept prototype for future optimization of the microreactor and toward providing sustainable solutions for access to clean drinking water.

Ultraviolet Photolytic Inactivation of *Cryptosporidium parvum*, *Bacteriophage MS2* and *Escherichia coli* within a Micro-Reactor

by
Christine C. Nguyen

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented July 9, 2019
Commencement June 2020

Master of Science thesis of Christine C. Nguyen presented on July 9, 2019.

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Christine C. Nguyen, Author

ACKNOWLEDGEMENTS

I could not have completed this project without the guidance, help and emotional support from Dr. Matthew Young Coblyn and Omar Mohamed. Thank you for being the best team I could ever asked to be a part of. There are no words that could express how much I appreciate you both. But I have loved every conversation, every laugh, every teaching moment, and all the understanding (and even both of your perpetual lateness). Thank you for always being supportive, encouraging, and the best part of my day. Working with you both has been one of the highlights of my master's thesis.

I would like to thank CBEE for welcoming me into this department and supporting me as their own. Thank you to the incredible friends in CBEE and lab mates that have been there to share knowing glances, bounce ideas off, inspire, support and help me through this all.

Thank you to my parents for giving me this opportunity to pursue higher education and for providing me with opportunities you did not have access to. You both have always been an inspiration for me to reach higher and succeed further.

I would like to thank my partner Cody Cullen. Thank you for enduring countless presentations of my work, helping me with teaching, logic processing, troubleshooting problems, double checking calculations and for always reminding me that its not the end of the world. I would not have been able to do any of this without your unwavering support and love.

Finally, I would like to express my deepest gratitude and sincerest appreciation for my advisor, Dr. Tala Navab-Daneshmand. Who has been more than an incredible advisor, role model, and mentor, but most importantly a friend. I am forever indebted to the lessons you have taught me, and the person you have molded me into. I will miss our shared interests, conversations and you dearly and will always be grateful for every opportunity you have provided me. I would not be the person I am today without you and am so lucky to have lived a life knowing you.

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1. INTRODUCTION

Modernization of human life by technological advancements has solved many problems in regard to quality of life. The development of vaccines, therapeutics, and water sanitation have been an essential step toward the shift from developing to developed nations. However, despite advances in sanitation, waterborne illness is still the leading cause of mortality in the world (World Health Organization, 2009) . Approximately 3.4 million people die yearly from waterborne pathogens such as *Salmonella*, *Cryptosporidium*, *Escherichia coli* (0157:H7), and *Legionella* (World Health Organization, 2009). Lack of access to adequate water and sanitation has contributed to an average of 1,980,000 deaths of children under five from diarrheal disease (World Health Organization, 2016). Reduction of these numbers can be accomplished by increasing access to proper sanitation, clean drinking water and hygiene practices.

In 2012, 2% of 748,000 annual cases of *Cryptosporidium* infections were reported within the United States (Painter, 2015). *Cryptosporidium* is one of the leading waterborne pathogens characterized by the CDC and is considered worldwide pathogen of concern (Pumipuntu, 2018), (Painter, 2015). Unlike some waterborne pathogens, prevalence and distribution of *Cryptosporidium* are not correlated to development status of the country it is found within. *Cryptosporidium* is a problem pathogen for both developing and developed countries; however, with more impact on the latter due to the lack of proper sanitation methods. Individuals with healthy immune systems can contract *Cryptosporidiosis* from water recreation, contact with infected animals or humans, or from drinking/exposure to contaminated waters (Painter, 2015). Symptoms usually include vomiting and diarrhea for 12-14 days but will eventually recover on their own. While those with compromised immune systems (e.g., AIDS, HIV, cancer, children

under 5, and adults over 65) can experience mortality. There are currently no drugs only hydration treatments to counteract the symptoms of diarrhea (Pumipuntu, 2018).

Conventional drinking water treatments such as chlorination are ineffective at disinfecting and inactivating *Cryptosporidium* due to a robust calcium shell (Hijnen, 2006). Proven methods require high intensity ultraviolet light to damage the parasite at a molecular level and prevent replication processes (Hijnen, 2006). Other methods for inactivation of *Cryptosporidium* are physical filtration, heat application, and ultraviolet radiation (Anderson, 1985) (Hijnen, 2006). Households without access to safe drinking water, who for example, use well water, or are in the state of an outbreak require a point-of-use treatment. Currently in the event of an outbreak, a boil-alert is issued and all water that is to be consumed or used/applied must be boiled to inactivate *Cryptosporidium* (Painter, 2015). Although some households have larger at home UV treatment systems, the development of a small, energy efficient, economically feasible and rapid method of disinfection is lacking. The use of microreactors and microscale technology has shown to improve process efficiency by exploiting intensified processes of diffusion, heat and mass transfer (DeWitt, 1999). A microscale-based technology reactor has been developed that would provide novel applications to conventional treatment methods as it is able to be installed at the point-of-use, directly into the tap line of a household. Due to the shallow channel depth, less light is attenuated through the water resulting in more efficient use of photons towards inactivation. Process intensification within microreactor processes make working with smaller quantities more feasible and therefore making operation safer (DeWitt, 1999).

Although our main objective of this research is to better understand *Cryptosporidium* parvum and its inactivation, we also evaluated the efficacy of *Bacteriophage MS2* and *E. coli* to

see if our device could meet the regulation standards provided by WHO (World Health Organization, 2016). Our overall objective with this reactor was to develop a proof of concept a point-of-use device that illustrates treatment of microbial pathogens. The successful application of a point of use device UV light to inactivate *Cryptosporidium*, *Bacteriophage MS2*, and *Escherichia coli* creates new possibilities for household drinking water treatment. Our specific objectives of this project are:

1. **Design, develop and implement a process for microbial inactivation utilizing ultraviolet radiation in a microscale reactor.**

2. **Determination of log-removal inactivation for *C. parvum*, *Bacteriophage MS2* and *E. coli* at varying residence times and constant concentration within a microfluidic reactor.**

LITERATURE REVIEW AND BACKGROUND

TREATMENT METHODS

Conventional Water Treatment Processes

Water treatment is divided into 3 high levels of treatment: primary, secondary and tertiary treatment. These levels are further subdivided into several intermediate processes: coagulation, flocculation, sedimentation, filtration and disinfection (Betancourt, 2004). Most treatment plants monitor for *Cryptosporidium* but do not actively target the parasite unless it exceeds allowable values (EPA, 2006). Common treatment methods employed by treatment plants based in metropolitan areas include disinfection via ultraviolet or ozone (Betancourt, 2004). *Cryptosporidium,* however, has been shown to be the most resistant of waterborne pathogens to chlorination methods (EPA, 2006). Studies have shown zero levels of inactivation after 18 hours of contact time with chlorine at high concentrations (Betancourt, 2004). An effective treatment, exposure to Ultraviolet light has been proven to be effective at inactivation at the molecular level (Morita, 2002).

For alternative treatment methods, metropolitan, rural and small communities have been able to integrate sand filters to treat wastewater naturally with biological, chemical and physical processes (EPA, 2006). For treatment plants that do not employ UV disinfection, physical separation by filters (e.g. membrane filtration, sand filters, etc.) are a viable method for controlling and mitigating exposure risks during a *Cryptosporidium* outbreak (EPA, 2006). Sand filter performance is consistent and have low operation, low maintenance and startup costs when compared to UV disinfection (Timms, 1995). *Cryptosporidium* is approximately 3-6 μM, larger than the standard bacterium at 1 μM, sand filters are able to physically separate the parasite out of the influent efficiently (Chapman, 1990).

Regulation Standards

The Environmental Protection Agency (EPA) regulates the Safe Drinking Water Act (SDWA) which requires an acceptable level of contamination to be defined per contaminant. The other alternative put forth by the EPA was to define a treatment technique per contaminant and the states are able to set more stringent regulations if they feel so (EPA, 2018).

For viral pathogens, the SDWA for drinking water regulations require systems to disinfect and filter their water for a 3-log reduction or inactivation of viruses (EPA, 2018). For *E. coli*, systems that yield a positive sample during a Total Coliform monitoring assessment are subjected to corrective action. Typically, these systems must demonstrate compliance of a 4-log removal of viruses to corrective a positive total coliform sample (EPA, 2018).

Regulations for *E. coli* and viruses have always been easily defined. However for *Cryptosporidium*, separate regulations and rules were created to mitigate risks from these pathogens. Under the Safe Drinking Water Act from the EPA, the Surface Water Treatment Rule 1989 (SWTR) removal of *Giardia* and Viruses were recognized at 3-log and 4-log requirements, with no removal requirements for *Cryptosporidium* for public water systems (PWS). In 1998, the USEPA amended the SWTR to include the "Interim Enhanced Surface Water Treatment Rule" (IESWTR) to place stricter regulations of *Cryptosporidium* levels in drinking water by monitoring microbial pathogens (Betancourt, 2004). The result of monitoring place PWSs into filtered system or unfiltered system requirements. For filtered systems, to improve control of *Cryptosporidium* it was mandated for disinfection to achieve at least 2-log removal (EPA, 2006). After the implementation of the Long Term 2 enhanced Surface Water Treatment rule (LT2ESWTR) requirements for log removal were determined based upon a filtration requirement for the treatment system. For example, public drinking water sources with levels of oocysts >1

oocyst/L (100 oocysts/100L) are required to provide a 4-log or 99.99% removal or inactivation of *Cryptosporidium* (Table 1) (Betancourt, 2004).

	Public Drinking water Source Levels	**Required Log Removal**
Filtration (n=0 oocysts)	n/a	2-log (99%) or inactivation of *C. parvum*
	>0.075 oocysts/L (7.5 oocysts/100L)	3-log (99.9%) or inactivation of *C. parvum*
	>1 oocyst/L (100 oocysts/100L)	4-log (99.99%) or inactivation of *C. parvum*
	>3 oocysts/L (300 oocysts/100L)	4.5- log (99.995%) or inactivation of *C. parvum*

Table 1. EPA Standards Enhanced Surface Water Treatment Regulations (ESWTR) for *C. parvum*

Under the LT2ESWTR, PWS's that employ filtration of their water are categorized into a bin for removal requirements. A filtered PWS can seek exemption from *Cryptosporidium* monitoring if the treatment can be validated to achieve 5.5-log reduction, typically by a UV disinfection system in addition to the filtration treatment (EPA, 2006).

Table 2. EPA Bin Requirements for Filtered PWSs

***Cryptosporidium* Concentration (oocysts/L)**	**Bin Classification**	**And if the following filtration treatment is operating in full compliance with existing regulations, then the *additional* treatment requirements are[2]...**			
		Conventional Filtration Treatment (includes softening)	**Direct Filtration**	**Slow Sand or Diatomaceous Earth Filtration**	**Alternative Filtration Technologies**
< 0.075	**1**	No additional treatment	No additional treatment	No additional treatment	No additional treatment
≥ 0.075 and < 1.0	**2**	1 log treatment[3]	1.5 log treatment[3]	1 log treatment[3]	As determined by the state[3,5]
≥ 1.0 and < 3.0	**3**	2 log treatment[4]	2.5 log treatment[4]	2 log treatment[4]	As determined by the state[4,6]
≥ 3.0	**4**	2.5 log treatment[4]	3 log treatment[4]	2.5 log treatment[4]	As determined by the state[4,7]

Table 2. EPA Bin Requirement for Filtered Public Water Systems (PWS) for *C. parvum* Inactivation

PWS's that do not employ filtration display exemption from filtration requirements due to the low-to-none influx of protozoan cysts found in their systems. They are required to achieve at least 3-log inactivation of *Giardia* and 4-log inactivation of viruses as mandated by the SWTR but employ a different set of requirements for *Cryptosporidium* inactivation (Table 3) (EPA, 2006).

Table 3. Requirements for Unfiltered PWSs

Average *Cryptosporidium* Concentration (oocysts/L)	Additional *Cryptosporidium* Inactivation Requirements
≤ 0.01	2 log[1]
> 0.01	3 log[1]

Table 3. Requirements for Unfiltered PWS's for *C. parvum* Inactivation

Unfiltered PWS can seek exemption from *Cryptosporidium* monitoring when a UV disinfection system is installed and validated for 3-log inactivation, the maximum inactivation treatment mandated by the LT2ESWTR for unfiltered PWSs) (EPA, 2006).

The World Health Organization (WHO)

Table 4. World Health Organization (WHO) International Scheme to Evaluate Household Water Treatment Technologies (WHO, 2016)

Performance Classification	Bacteria (log reduction required)	Viruses (log reduction required)	Protozoa (log reduction required)	Interpretation
3	4-log or more	5-log or more	4-log or more	Very high pathogen removal
2	2-log or more	3 log or more	2-log or more	High pathogen removal
1	Meets at least level 2 performance classification for 2 classes of pathogens			Targeted Protection

The World Health Organization has developed an international scheme to evaluate household water treatment technologies. The technology in question must at least meet requirements for 2 out of the 3 microbes to be eligible implemented into homes. Past this requirement, there are higher performance classifications for technology as seen in Table 4.

Alternative Methods of Treatment

During outbreaks of *Cryptosporidium*, the recommended treatment of water for immediate use is to boil water (Chyzheuskaya, 2017). Heat has been shown to be an excellent inactivator of *Cryptosporidium*. Studies have indicated a direct relationship between contact time of higher temperatures and levels of infectivity reduced within the parasite (King, 2005). At higher temperatures contact time is reduced but parasites are still rendered non-infectious. When exposed to temperatures such as 73.7 °C for a contact time of 5 seconds or longer, or 72.4°C for 1 minute or longer, the parasite has been proven non-infectious and inactivated (Anderson, 1985) (King, 2005). At lower temperature values compounded with longer contact times yield the same results: 50-55°C for 5 min, or 64.2 for 2 min or longer (King, 2005). Temperature increase lead to a direct correlation to oocysts infectivity decreasing (King, 2005).

Infectivity is maintained by the robust and multilayered oocyst wall (King, 2005). The ability to be infective is a sign of metabolic capacity as the carbohydrate reserves in the form of amylopectin are consumed in direct response to ambient environmental temperatures rendering infectivity as a metabolic function (King, 2005). Temperature plays a direct role in disintegrating the oocyst calcium wall and exposing the cysts within the wall to unfavorable conditions. Temperature dependent inactivation of oocysts is a function of metabolic ability (Fayer, 1994).

Oocysts were found noninfectious at 64.2 °C or higher for 2 minutes or longer and at 72.4°C or higher for 1 minute (Fayer, 1994).

Protozoa - *C. parvum*

Biology and Morphology

Cryptosporidium is an apicomplexan parasite that infects the microvillus border of either the gastrointestinal tract or the intestines. Apicomplexan parasites are a phylum of parasitic alveolates whose defining trait is the possession of an organelle containing an apicoplast that allows the organism to penetrate a host cell (Xiao, 2004). Microvillus are small protrusions along organs that increase surface area while decreasing volume and facilitate functions of absorption, cellular adhesion, and secretion. *Cryptosporidium* burrows itself into the microvillus to gain access to either the gastrointestinal epithelium or the intestines where it finds optimal living conditions.

There are approximately 26 named species, which are taxonomically categorized by the host they infect (Xiao, 2004). *C. parvum* infects specifically cattle, sheep, goats, deer and humans. Because different serotypes target different hosts, it is important to note that not all species of *Cryptosporidium* have the same capacity of infectivity (Xiao, 2004). *C. parvum* is the most commonly reported species within mammals and has been confirmed to infect the microvilli of the small intestine and the colon. *Cryptosporidium* live monoxenous life cycles within the host's infected organ. Because it is an apicomplexan, *Cryptosporidium* burrows itself into a closed compartment of the plasma membrane known as the parasitophorous vacuole, allowing it to develop within the host protected from phagolysosomes (Thompson, 2005). The parasitophorous vacuole provides optimal conditions for the parasite to thrive, presenting it

protection from the gut environment, while energy and nutrients are provided via an apicomplexan specific organelle (Tzipori, 2002).

Life Cycle

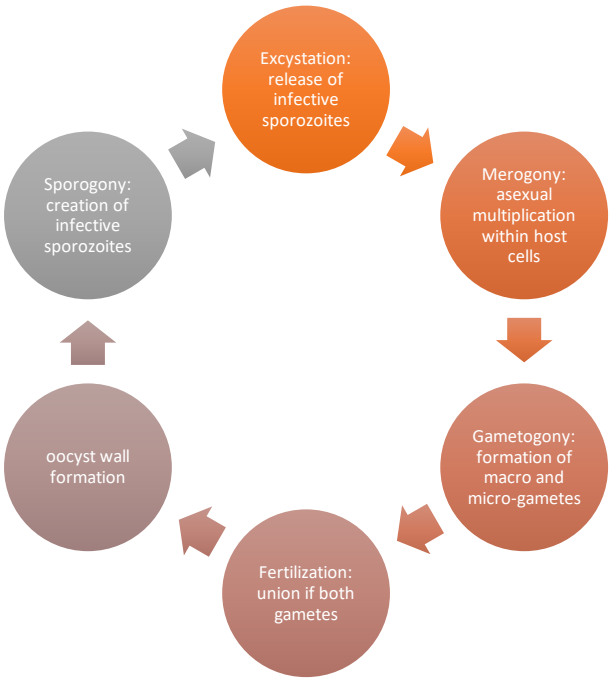


Figure 1. *Cryptosporidium* Life Cycle: Divided into six major developmental phases: Merogony, Gametogony, Fertilization, Oocyst wall formation, sporogony and Excystation

Upon ingestion the stomach acid and bile salts destabilize the oocyst wall and result in the release of four sporozoites that invade the host (Figure 1). The sporozoites burrow into target host cells (stomach or intestine depending on serotype) and differentiate into trophozoites (Pumipuntu, 2018). Trophozoites create merozoites which can invade the host cells and produce micro-and-macrogametes that undergo sexual reproduction (gametogony) (Xiao, 2004) . Mating in *Cryptosporidium* occurs between proximal siblings which results in a clonal structure population. Oocysts reproduced have two types of walls: thin and thick oocysts (Barta, 2006).

Thin walled oocysts will stay in the gastrointestinal or intestinal tract while thick walled oocysts will be shed alongside feces (Bouzid, 2013). The thin walled oocysts have the ability to be re-infective to the host is unable to shed all lodged cysts (Painter, 2015).

Metabolic Adaptations

Cryptosporidium, specifically *C. parvum* (Iowa) and *C. hominis* (TU502) have undergone extensive genome sequencing analysis (Xiao, 2004). It was found that contrary to most apicomplexans, *Cryptosporidium* contained only necessary cellular structures and lacked both apicoplasts and mitochondria (Xiao, 2004). In accordance with optimizing energy balances within the organism, *Cryptosporidium* was found to derive its metabolism from glycolysis but also had the capabilities of generating metabolic pathways during aerobic and anaerobic situations allowing environmental flexibility when conditions are not optimal (Barta, 2006). Although *Cryptosporidium* is nestled in the plasma membrane, it is able to utilize multiple transporters to facilitate nutrients and from the host as it has limited biosynthetic capabilities (Rider, 2010).

Quantification

Cryptosporidium is not culturable and must be quantified with microscopy accompanied with fluorescence dyes, histology, or molecular methods (Morita, 2002). Clinical detection of *Cryptosporidium* can be performed directly from a stool sample smear with fluorescence microscopy or histological methods (EPA, 2005). However, further identification of species subtype must be quantified with molecular methods (i.e. DNA extraction, PCR, sequencing, etc.) (Bouzid, 2013).

Viability assessments for *C. parvum* are the most commonly employed by vital dye inclusion or exclusion assays or in-vitro excystation procedures. Both procedures focus primarily on the permeability of the cell wall (King, 2005). In vital dye staining, a live and dead stain are applied in dual incubation to the sample. Following incubation periods, cells that fluoresce blue in standard brightfield microscopy are considered "viable" or "alive" and those that fluoresce red are "non-viable" or "dead"(Campbell, 1992). The terms "viable" and "non-viable" are subjective to each paper. There is no standardized definition in viability staining of either word nor of the connotations of "live" and "dead" (Netuschil, 2014) .

Infectivity Quantification

Vital-dye based assays provide surrogate indicators of oocyst permeability or response to biological stimuli (Campbell, 1992). Utilization of these dye assays for multiple research applications have contributed to confusion when analyzing the data from these assays. Excystation is an in-vitro method for *Cryptosporidium* enumeration and a valid method of accessing viability (Ryan, 2015). It is performed by exposing oocysts to bile salts or specific surfactants followed by a 37°C incubation to release infective sporozoites (Campbell, 1992). Unfortunately, it has been found to consistently overestimate the viability of *Cryptosporidium* oocysts and is not the most reliable method for viability studies (Black, 1996). Infectivity quantification is most successful from cell-culture *in-vivo* studies with mice (Slifko, 1997).

Viruses – *Bacteriophage MS2*

Bacteriophage MS2 is a viral indicator used to assess the effectiveness of treatment systems for pathogenic viruses such as norovirus, rotavirus, and enteroviruses (Furiga, 2011). *Bacteriophage MS2* is often used in water quality research as a surrogate for viruses because it shares the same ultraviolet inactivation mechanism as both *E. coli* and *C. parvum*. (EPA, 2006). *Bacteriophage MS2* is an icosahedral virus with a single RNA genome and is an F-specific RNA virus that infects by attaching its receptors to the F-pili of *Escherichia coli* (Wigginton, 2012). Upon entrance to the host cell, *MS2's* proteins are created leading to the formation of *MS2* RNA transcription within the host (Beck, 2016).

Bacteriophage MS2 experiences a loss of viral infectivity due to direct RNA damage when exposed to ultraviolet radiation at the germicidal range (Beck, 2016). The mechanism of inactivation is identical to both *C. parvum* and *E. coli*, except because it contains RNA and not DNA a uracil-adenine dimer is created as opposed to adenine-thymine which is typically found in DNA based microbes (Wigginton, 2012).

Bacteria – *E. coli*

E. coli is a gram-negative bacterium commonly used as fecal contamination indicator in water systems. *E. coli* is approximately 0.5-1 μm wide, is susceptible to both heat and ultraviolet inactivation, and is typically found in the gut of health animals and humans (Blount, 2015). Many strains of *E. coli* are non-pathogenic while other harmful strains can produce shiga-toxins which cause gastroenteritis associated illnesses (Asakura, 2019). *E. coli* shares the same ultraviolet radiation inactivation kinetics as *Cryptosporidium* and *Bacteriophage MS2 (Hijnen, 2006).*

MICROFLUIDICS

Micro-technology is defined as systems fabricated with dimensions within the sub-millimeter range with diameters ranging from 100 to 1000 microns and 1to 10 mm in length (Burns, 1999). Micro-reactors are miniaturized systems that intensify mass and heat transfer processes by possessing incredibly small internal volumes (DeWitt, 1999). Chemical reactions occur within the micro-reactor often within micro-channels and have several advantages to traditional systems (De Sa, 2016).

Some of these advantages are that variables like mass and heat transfer, temperature, pressure, and diffusion parameters are intensified and are therefore, increased within these systems (Jensen, 1999). Because microreactors have such small dimensions, assumptions of laminar flow can be made allowing easy quantification of heat and mass transfer (De Sa, 2016). Studies have confirmed that laminar flow profiles allow for rapid mixing and therefore, increase mass and heat transport (Beebe, 2002). Increases in mass and heat transport have been shown to be a factor of 100 times faster when scaled 100 down orders of magnitude (DeWitt, 1999). This increase allows for faster processing of samples and higher throughput of sampling with microtechnology (Mampallil, 2012). With these parameters, chemical kinetic parameters are easily extracted and can be combined with modeling software for optimized reaction parameters. Optimization of reaction processes decreases the amount of experimental work required increase efficiency with respect to time and labor of the operator (De Sa, 2016). Microfabrication reduces utilization of expensive reagents and components with smaller volumes and can also reduce fabrication costs with “numbering-up” microreactors to increase production size and reduce time expenditure (Jensen, 1999). Here, “numbering-up” refers to the potential stacking or combination of multiple reactors in use (Sugimoto, 2006). Failure of micro-reactor systems pose untraditional

advantages, as reduced volumes within the microreactors are more easily contained than those of large-scale reactors and their respective larger volumes (Sugimoto, 2006).

CHAPTER 3: ULTRAVIOLET LIGHT

Properties of UV

Ultraviolet light at the germicidal range is achieved by utilizing a mercury vapor gas mixture because it emits light at the 254 nm range (EPA, 2006) (Figure 2). Where the concentration of the gas mixture is directly correlate to the pressure of the vapor and therefore the pressure of the light. UV lights can be categorized by pressure: low pressure (2×10^{-5} to 2×10^{-3} pounds per square inch (psi)), medium pressure (2-200 psi) (EPA, 2006). An increase in pressure of the lamp corresponds to an increase in temperature of the lamp as well; operating temperatures at higher intervals allow for increased collisions between the vapor atoms (at 254 nm, the gas is mercury) which results in a greater intensity of light produced by the lamp (EPA, 2006).

Characteristics of light

As light travels through a system it can be scattered, refracted, or reflected by particles or different materials present in the system (EPA, 2006). As light passes through a substance it can be absorbed and converted into heat, chemical energy, or can be shifted to alternative wavelengths (EPA, 2006). Once that light is transformed there is no UV light left available to inactivate pathogens (EPA, 2006).

While absorption is a transformation of light energy into another form of energy there are physical properties of light that can alter the direction of the photon's movement. Refraction occurs when the direction of the light is changed when it moves through the interface of mediums (EPA, 2006). Refraction plays an integral part in the angle at which UV light and the target pathogen interact. Reflection is another physical property that occurs when light is deflected off a surface and moves in a different direction. Reflection can decrease the intensity of the light on the desired surface and is dependent on the material of the surface it comes into contact with (EPA, 2006). Scattering is the last physical property of light where particles of light interact with particles and cause the light to scatter in multiple directions (including back toward the light – backscattering) (EPA, 2006).

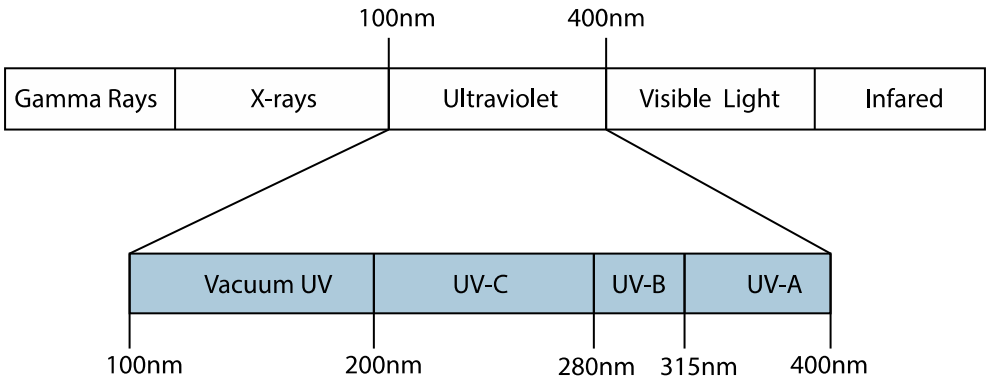


Figure 2. Electromagnetic spectrum wavelength distribution; Ultraviolet radiation wavelengths highlighted in blue from 100-400 nanometers

There are two major classes of UV radiation that can cause mutagenic damage. UV-C (200-280 nm) and UV-B (280-320 nm) distort the DNA helix by adding a 7-9 degree kink (Thoma, 1999). The degree of damage from UV radiation depends on the flexibility of the DNA, therefore sites that are more like to unwind or bend will be more favorable for damage

(Thoma, 1999).

Pathogen Inactivation

Inactivation by UV radiation repair can occur in two separate occasions, in the presence of light, photoreactivation, and in the absence of light, dark repair (Hijnen, 2006). In photoactivation, microbes can recover activity by repairing the dimers created when exposed to near-UV light at the 310-480 nanometer (nm) range by a photolyase enzyme (Oguma, 2001). During dark repair, enzyme photolyase repairs DNA damage without the presence of light (Morita, 2002 #28). It is confirmed that *C. parvum* can perform both types of photo-repair; however following repair infectivity of oocysts subjected to inactivation were not regained even though the dimers were repaired (Oguma, 2001). There exists no phenotypic difference between infectivity after photoreactivation or dark repair; suggesting that a separate mechanism is responsible for infectivity status (Hijnen, 2006). Infectivity status is best measured with infectivity assays which are performed with cell culture combined with molecular methods (Di Giovanni, 2005).

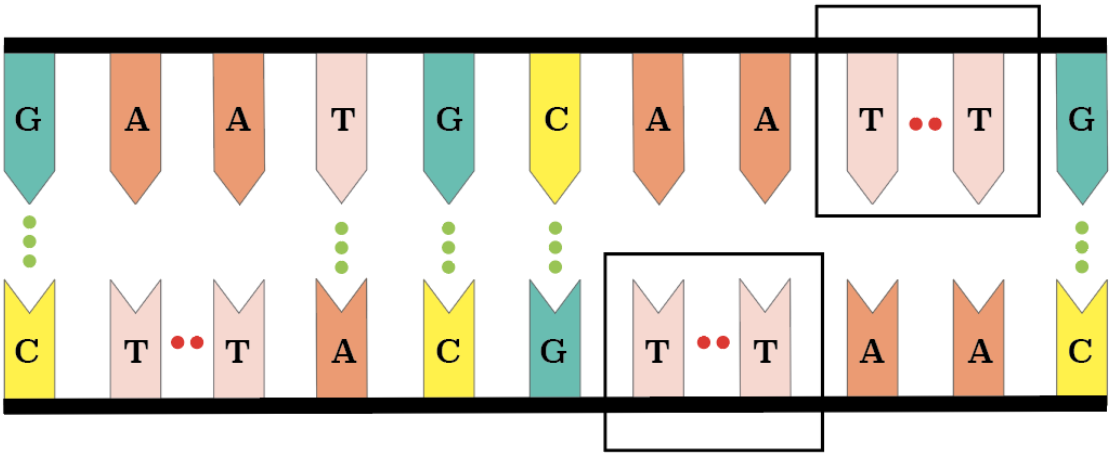
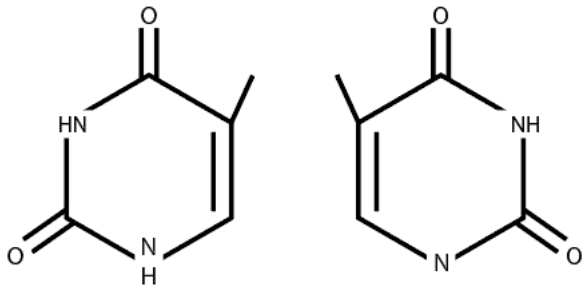


Figure 3. Formation of dimer lesions by UV Damage to DNA Strand

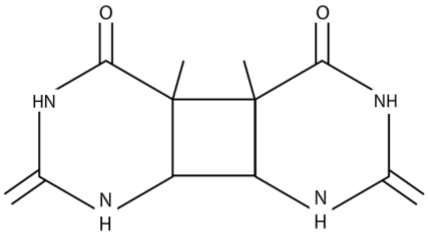
Inactivation by UV radiation is a molecular process that disrupts cell replication and transcription (EPA, 2006) (Figure 5). Where normal bonding should occur between pyrimidines and purine pairs, formation of dimers are created (Oguma, 2001) (Figure 3). Dimers are molecular lesions formed at the nucleic acid bases within DNA from photochemical damage (induced by the UV radiation) (Morita, 2002).

There are 6 different types of photochemical damage that UV can induce. Cis-syn cyclobutene-pyrimidine dimers (most common) and pyrimidine (6-4) pyrimidine photoproducts are the most prevalent in microbial disinfection (Ravanat, 2001) (Figure 4). Where the degree of damage from UV Radiation depends on the flexibility of the DNA in which sites that are more likely to unwind or bend are more favorable for damage (Thoma, 1999) .



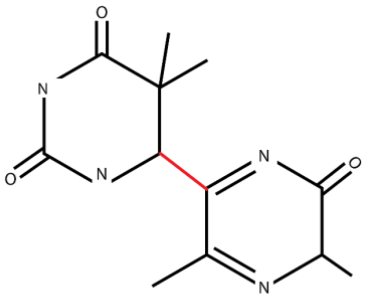
Regular thymine molecules

a)



Cis-Syn Cyclobutane Dimers

b)



6-4 photoproduct

c)

Figure 4. Dimers created from ultraviolet photochemical damage a) Non-damaged adjacent thymine molecules, b) photochemical Cis-Syn Cyclobutane Dimer, c) 6-4 photoproduct dimer

Covalent bonds are created between adjacent nucleotides and disrupt normal bonding patterns of the nucleic acids (EPA, 2006). After a significant number of these lesions are made, they can inhibit replication enzymes such as DNA polymerase and can cause deviations in transcription and replication processes coding for eventual cell death (Morita, 2002).

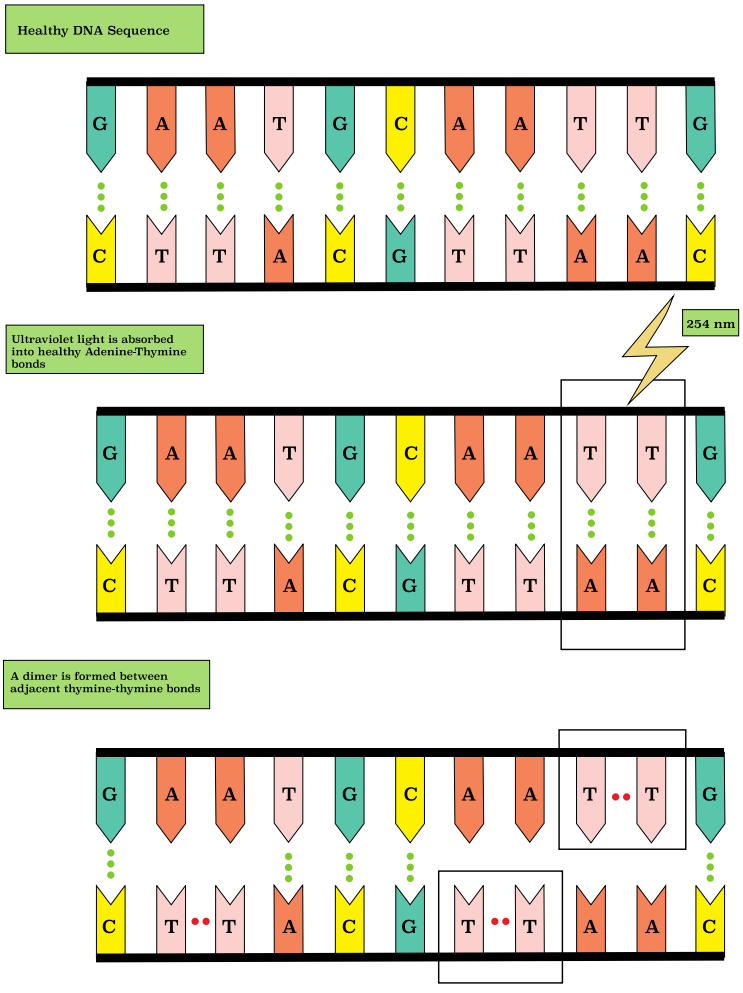


Figure 5. Complete Molecular Mechanism of DNA inactivation by UV Radiation by formation of dimers. A healthy DNA sequence is exposed to ultraviolet light, the Adenine-Thymine nucleic acids absorb the light energy at 254 nm. The bond breaks and forces the dimerization of an adjacent thymine molecule.

Inactivation of microorganisms is defined as the reduction of an initial concentration of microorganisms due to exposure of a disinfectant at a specific contact time (Hijnen, 2006). Inactivation can be quantified by:

$$\text{Log inactivation} = \text{Log}\left(\frac{N_0}{N}\right)$$

Equation 1. Log Inactivation Equation (EPA, 2006)

Where N_0 is the concentration of microorganisms in the influent (pre-treatment) and N is the concentration of microorganisms in the effluent (post-treatment).

Ultraviolet light has many advantages for protozoan disinfection. UV light requires short contact times between microbes and light, has no known disinfection byproducts and that it is a physical process and requires no additional chemical inputs (Betancourt, 2004). There are few disadvantages to UV radiation to treat *Cryptosporidium* however they are significant. UV treatment can be effective in inactivating *Cryptosporidium*, however, at UV doses much higher than typical ranges in water treatment (EPA, 2006 #1). UV radiation does not produce harmful byproducts unlike traditional chlorination within wastewater (Betancourt, 2004). Many microbes are inactivated on a molecular level by UV radiation by the formation of lesions on the DNA. These lesions inhibit DNA replication by formation of mis-matched amino acid pairings called dimers (EPA, 2006). *Escherichia coli* is often used as control for *Cryptosporidium* as their susceptibility to UV radiation is nearly identical (Hijnen, 2006). Both organisms respond in a similar fashion to UV radiation, as their cell walls are ineffective at resisting radiation. Similar to human biology, microbes are able to repair UV induced damaged to their DNA. Two mechanisms exist for DNA reparation for *Cryptosporidium*: photoreactivation (light repair) and dark repair (Oguma, 2001). When exposed to the polychromatic for a specific contact time,

Cryptosporidium parvum is able to harness the energy from the visible light and repair dimers in its DNA. Dark repair can occur with the enzyme photolyase and correct for photoproduct dimers within the DNA (Oguma, 2001).

CHAPTER 4: VIABILITY VS. INFECTIVITY QUANTIFICATION OF C. PARVUM

Vital-dye based assays provide surrogate indicators of oocyst permeability or response to biological stimuli. Utilization of these dye assays for multiple research applications have contributed to confusion when analyzing the data from these assays. There exists confusion between the purpose of viability assays and infectivity assays within *Cryptosporidium parvum* research. The purpose of this paper is to highlight and assess the reasons why vital dye assays are not appropriate for infectivity quantification. This chapter addresses that (i) viability is an indicator of cell wall permeability and not that of metabolic activity which is the chemical driver of infectivity, (ii) papers that quantify infectivity do so with cell culture or animal models, not viability assays, even though *C. parvum* has been widely cited to be non-infective post treatment, (iii) research studies that utilize infectivity assays are able to quantify inactivation however vital dye papers are able to quantify inactivation but not infectivity, (Chyzheuskaya et al.) it has been explicitly stated that viability and infectivity are not correlated, as vital dyes provide a severe misestimation of viability.

Viability assessments for *Cryptosporidium parvum* are most commonly employed by vital dye inclusion or exclusion assays or in-vitro excystation procedures. Both procedures focus primarily on the permeability of the cell wall. In vital dye staining, a live and dead stain are applied in dual incubation to the sample. Following incubation periods, cells that fluoresce blue in standard brightfield microscopy are considered “viable” or “alive” and those that fluoresce red are “non-viable” or “dead”. (Campbell et al.) The terms “viable” and “non-viable” are subjective to each paper. There is no standardized definition in viability staining of either word nor of the connotations of “live” and “dead”. (Netuschil et al.)

In-vitro excystation methods quantify viability utilizing statistical most probable number (MPN) calculations in conjunction with fluorescence microscopy. An acidifying bile agent and sodium hydrogen carbonate are most commonly added to the sample, mimicking a host stomach environment. Once exposed to the bile and acid, the outer shell of the oocyst disintegrates allowing the four sporozoites inside to be released into the environment. (Campbell et al.) In-vitro excystation methods compare and contrast the number of oocysts prior to excystation to the final number of shells and excysted organisms in the sample. Differences are noted and computed with a widely accepted mathematical formula. (Slifko et al.)

While both studies in the past have noted that these assays demonstrated potential to describe viability, they are inherently based on the condition of the oocyst shell. Permeability dyes work by intercalating with the double stranded DNA helices. Where vital dyes concerned, certain parameters such as membrane integrity are dependent on stress conditions applied to the cell. Propidium iodide (PI) is a common fluorescent agent that stains compromised cells as a result of a now porous membrane. PI cannot physically enter viable cells or dead cells that have intact cell membranes and is used as a surrogate indicator of compromised viability. (Kainz et al.) Fluorescent stains like 4', 6-diamidino-2-phenylindole (DAPI) are non-specific dyes that bind to the minor grooves of the adenine-thymine regions on DNA and allow easy visualization of the nucleus. DAPI is able to pass through the cell membranes of live cells and will also act as a surrogate indicator of viability. (Chazotte et Al.) In current research pertaining to *C. parvum*, the use of viability dyes are surrogates for rapid detection and examination in research studies. (Netuschil et al.)

The infective agent of the oocyst are sporozoites, shielded from environmental conditions by the thick-walled shell. Infectivity is a product of metabolic activity and cannot be verified by

dye permeability assays. There is no enzymatic cleavage prior to the stain's fluorescence, DAPI exposure to the DNA occurs readily after it passes through the oocyst wall. (Jenkins et al.) The case of infectivity and metabolic activity has been corroborated in many studies. Another experiment demonstrated the correlation between oocyst infectivity and oocyst ATP content with the main concept behind the study being that energy production and expenditure is required for infection and can be quantified by measuring ATP. (King et al.) By measuring oocysts over several environmental temperatures, it was found that the depletion of energy reserves changed over time when temperatures increased. A cell culture-TaqMan PCR assay was utilized for quantitative, sensitive and reproducible measurement of oocyst inactivation by disinfection. This study in particular looked at temperature fluctuations as the main method of disinfection and found that with an increase in temperature oocyst infectivity decreased. (King et al.)

It was determined that the ability to be infective is indeed linked to the carbohydrate energy reserves in the form of amylopectin found in *C. parvum* which is consumed in direct response to ambient environmental temperatures. When temperature variables were increased, a correlation of utilized amylopectin was defined, indicating that *C. parvum* infectivity is linked to the finite carbohydrate energy stores within the cell. (King et al.) It is important to note that quantification of amylopectin stores can be used as a surrogate marker for infectivity, similar to the way that vital dyes are used for viability. However, there are unknown external parameters regarding *C. parvum* metabolism, therefore we cannot assume amylopectin is the principal energy source within the parasite. *C. parvum* has lipids and crystalline proteins within its system. During quantification, amylopectin granules from broken oocyst shells can secrete excess amylopectin stores making quantification inaccurate.

Glycolysis is the principal source of energy in coccidia, where the breakdown of lipids entering this pathway can convert glycerol into other compounds. (Bukhari et al.) Measurement of infectivity via amylopectin stores would underestimate energy reserves for long periods of time within *C. parvum*. In order to quantify infectivity to metabolic capacity, a luciferase reaction was utilized to ascertain an accurate measurement of total metabolic energy. This measurement was regarded as the actual potential for sporozoite infectivity as opposed to amylopectin content, quantification of specific metabolites or other enzymatic processes occurring within the cell. It was further quantified that when disinfection processes were applied, the cell culture exhibited a decrease in infectivity when the samples had been exposed to higher temperatures for longer durations of time. This indicates that infectivity is a function of metabolic activity and not of the permeability of the cell wall. (King et al.)

Viability and inactivation evaluated by cell culture state that oocyst survival in the environment utilize "indicators" such as "vital dye staining" or "in-vitro excystation". These statements are carefully noted to be only indicators and have been known to misestimate animal infectivity. Several papers have exclusively stated that the only method appropriate for evaluating infectivity are animal or cell culture models. (King et al.), (JENKINS et al.), (Matsubayashi et al.), (Morita et al.), (Rochelle et al.)

It has been determined that there is no phenotypic difference between cells who do or do not have infective potential; illustrating that a viability test assumes "viable" confers infectivity would not truly reflect the actual infectivity of the sample. (Morita et al.) Another experiment compared four in-vitro surrogates for viability to animal infective models and determined that there were statistically significant differences between the two. Their research affirmed that

infectivity assays via animals followed by cell culture should still be regarded as the "gold standard" for infectivity determination in disinfection studies. (Bukhari et al.)

It was noted that among the four in vitro methods tested, including vital dyes and in vitro excystation, there were large differences. Where consistent differences were detected between animal infectivity models and in-vitro viability assays. The in-vitro assays were shown to have misestimated viability in comparison to animal models. To directly compare the viability assays with animal models, a study exposing oocysts to the same disinfection method was separated into two groups. After the exposure to the disinfection, viability and infectivity experiments were performed and indicated that viability assays showed consistently different viability values than those indicated by infectivity assays. The study was able to confirm that viability dyes do not estimate oocyst viability to the same order of magnitude when compared to animal infective models which has been corroborated by many other researchers ((KORICH et al.), (Black et al.), (FINCH et al.)).

It is important to note that all studies referenced that employ infectivity assays within their experiment utilize cell culture or animal infective models for quantification and not vital dye assays. The dye assays were unnecessary in these experiments as cell culture methods combined with PCR were able to quantify inactivation and infectivity. Vital dye assays are crucial to studies in which a rapid detection method is required. For surrogate indicators, vital dye assays are useful for estimates of viability but ineffective at quantifying infectivity. Studies that incorporate infectivity determinations within their research, do not demonstrate use of vital dyes even though it is widely accepted that post treatment the oocysts are non-infective. Research utilizing vital dyes often quantify inactivation which is unknown if it is an accurate representation of infectivity; however those studies are also caveated with a blanket statement of

surrogate indication. It is necessary to perform accepted, widely-used methods of infectivity quantification for *Cryptosporidium parvum* when disinfection treatment is of interest. Under or overestimation of *Cryptosporidium parvum* values in treatment methods can provide insufficient technology development and compromise public health.

Chapter 5: RISK ASSESSMENT

Cryptosporidium is an opportunistic waterborne protozoan parasite, categorized by the Center of Disease Control as one of the leading causes of waterborne illness in the United States. Incidence of cryptosporidiosis, the gastrointestinal illness caused by the chlorine-resistant parasite, are estimated to be 748,000 cases annually with an approximated $45.8 million dollar cost per year in hospitalizations (Painter, 2015). There are currently no small scale at-home treatment procedures and wastewater treatment plants that do not employ UV radiation or sand filter physical separation are at risks for outbreaks as standard chlorination disinfection is not a viable method for controlling *cryptosporidiosis* (Gale, 2000).

Cryptosporidiosis and Human Health Concerns

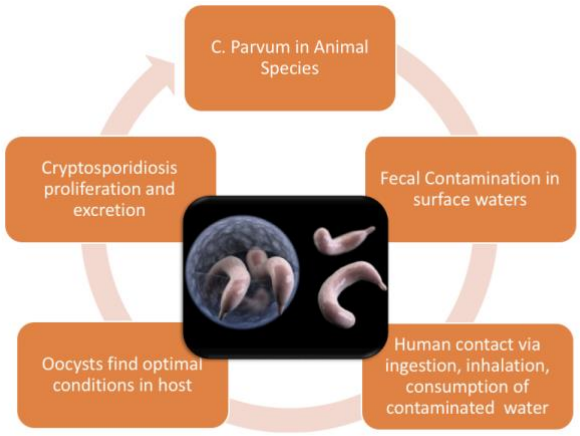
When *cryptosporidiosis* is reported, symptoms include weight loss, abdominal pain, anorexia, fatigue, fever and vomiting, and profuse diarrhea. However, an overwhelming, two-thirds of *cryptosporidiosis* infections are not reported. For healthy individuals, the disease is self-limiting, and individuals resume normal bodily functions within a two-to-three-week time frame (Bouzid, 2013). However, for immunocompromised individuals, such as those with cases of human immunodeficiency virus (HIV), aids, autoimmune disease, and children with susceptible immune systems, the disease can be life-threatening and fatal (Painter, 2015). Children under the age of 5 can acquire “short-lived immunity” or “incomplete” in which they survive the initial infection but are still susceptible to *Cryptosporidiosis* later in their lives. Severity of virulence is also dependent on the specific host’s degree of immune suppression. For individuals with impacted T-cell function (AIDS/HIV candidates) the risk has been found to be the highest (Hunter, 2002).

Extra-intestinal *cryptosporidiosis* occurring in the respiratory tract or pancreas have been reported in immunocompromised individuals enhancing mortality rates in this demographic as there is no cure for *cryptosporidiosis*. Virulence, symptoms and duration of infection is dependent of the species of *Cryptosporidium*, and the status of the victim (i.e. age, immune status, etc.) Serotypes of *Cryptosporidium* are also dependent on location and seasonal variation [Bouzid], however *C. parvum* is associated with more than 90% of human diarrheal cryptosporidiosis cases (Caccio, 2005). At this time there are no determined virulence genes for *Cryptosporidium* nor are there therapeutics or treatments for infected individuals.

Because virulence, symptoms and pathogenicity are dependent on species, the necessity for species determination of *C. parvum* to evaluate human health concerns is necessary. Taxonomy of *Cryptosporidium* is a concern for human health as when oocysts are determined in water sample detection, samples are not examined explicitly for species. Because virulence and pathogenicity are dependent on strain, taxonomy must be developed that can clearly differentiated between species to further evaluate risk to public health (Xiao, 2004). Because *cryptosporidiosis* has a high rate of fatality for immunocompromised individuals, it has been categorized as a concerning opportunistic pathogen that requires immediate attention to prevent massive global health complications.

Transmission

Transmission of *C. parvum* can occur directly or indirectly by animal-to-human (zoonotic), fecal-oral route from infected hosts, animal-to-animal, human-to-human and contact with contaminated objects, inhalation aerosolized species or environments, respectively (Bouzid, 2013). Both children and the elderly face common routes of infection such as direct person-to-person contact in daycare centers and nursing homes effectively transmit the disease. For most adults, the most common vector into the body is through food or waterborne route. For individuals living in rural areas, the main method of distribution is postulated to be through animal husbandry or ingestion of contaminated well-water. Infected individuals experiencing symptoms may pass the parasite to healthy individuals by practicing unsanitary hygiene habits or swimming in recreational areas open to public. Other ingestion risk factors reported are contact with infected individuals, international travel, contact with livestock, ingestion of recreation, drinking water, with outbreaks stemming from foodborne, animal-to-human, and person-to-person exposure routes (Gale, 2000). The number of oocysts ingested in order to cause infection has been reported to be less than 10 oocysts, while number of oocysts shed from excretion of feces is around 10^7-10^8 per bowel movement (Gale, 2000). Although *Cryptosporidium* is typically spread through contact with animals, in metropolitan areas it has been seen that transmission from humans may actually be more prevalent than animal transmission in infected spaces (Xiao, 2004). Hunter et al. concluded major risk factors for contracting *cryptosporidiosis*



from different species were different; for *C. parvum* exclusively animal husbandry lead to higher incidences of infection whereas *C. hominis's* risk factors were observed in direct contact with humans (Hunter, 2004).

On an economic front, damages to business and human health caused by *Cryptosporidium* are costly to mitigate. Individuals afflicted by cryptosporidiosis typically take 9-11 days off work, resulting in no compensation for their time, in addition to the caregivers who also take uncompensated time off work (Painter, 2015). Infrastructure not equipped to effectively handle *Cryptosporidium* outbreaks face approximately between $22.44 and $96.2 million dollars in outbreak costs, depending on the severity of the outbreak (Chyzheuskaya, 2017). Additional costs accrued from the general public can be broken down into these categories: private and public-sector costs, sick person costs, cost to business, costs of missing school/college, tourism costs, and care industry costs.

In the private sector costs to afflicted individuals consist of: purchasing bottled water and the cost of boiling water for household uses. For sick persons, self-medication of antidiarrheals, physician visits, and loss of income from missed work days by caregiver and infected individuals are identified (Painter, 2015). The cost to business and tourism industries are felt from sick leave by workers, productivity loss from lack of workers, reservation cancellations, extra boiled and bottled water to customers, and a reduction in travelers purchasing goods from local merchants (Chyzheuskaya, 2017). Care centers catering to the elderly and young experience similar costs as the private sector and must ensure their water is safe due to the condition of the immune systems of their constituents. Public sector costs include detection and culture tests, government regulatory agency costs, and treatment costs (Painter, 2015).

A brief table is listed below with a summary of different stakeholders and their respective loss characteristics during a standard *Cryptosporidium* outbreak (Chyzheuskaya, 2017) (Table 5). Total medical costs for mild, moderate, and severe illness characterization for an outbreak can average approximately $31,655,000 and productivity loss $64,589,000, accruing to a total loss of 96,244,000 (approximated for an outbreak population of 403,000 based off the US Milwaukee outbreak in 1993) (Corso, 2003).There is a necessity for safe and effective treatment of *Cryptosporidium* in drinking water systems. Technology that is able to detect, treat, and prevent potential infection of *Cryptosporidium* that will be able to mitigate extensive costs to both the general public and the government.

Group	Characteristic of Loss
Private Sector All Households: Hygiene, household use (cooking, washing) and consumption	Cost of extra bottled water Cost of boiling water
Sick Persons Reported and Non-reported individuals with cryptosporidiosis	Physician visits Self-medication of antidiarrheals Loss of income (work missed) Loss of income for caregivers (work missed)
Costs of Missing School/College	Average of 5-9 days taken off
Cost to Businesses and Tourism Productivity loss	Loss of workers Loss of production Cancellations Extra boiling water Extra bottled water Reduction in tourist purchasing
Care Industry Elderly Children Public Sector Costs	Cost of extra bottled water Cost of boiling water Loss of workers Hospital Costs Emergency department costs EPA/Regulatory Agency costs Identification, detection, culture tests
Hospital admissions	Cost of labor Response Team costs

Table 5. Summary of Stakeholder loss from *Cryptosporidium* outbreak adapted from (Corso et al.)

CHAPTER 6: MATERIALS AND METHODS

Justification of Design

The parameters of this microreactor were designed for photodegradation. The first-generation of the reactor was designed to maximize the flux of photons into the water stream regardless of target pathogen (Figure 6). A microreactor with a shallow serpentine channel was chosen to minimize UV light attenuation through channel depth while increasing residence time.

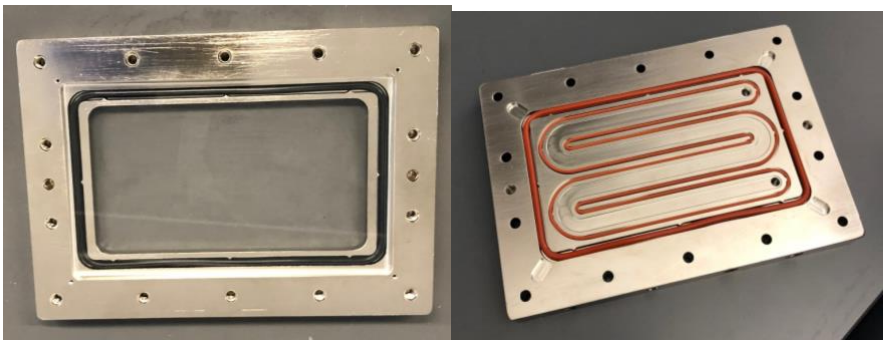


Figure 6. UV Microreactor taken unscrewed with components inside. A) Left side: Top plate with O-ring and quartz plate in window, B) Right side: Bottom plate with serpentine channel with inner O-rings

Experimental Apparatus and Methods

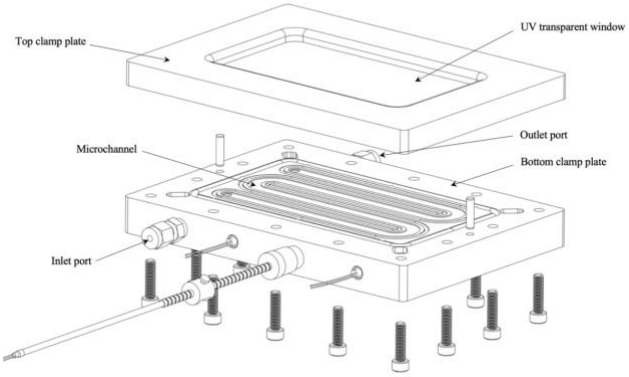


Figure 7. CAD Schematic of Microreactor Components

Materials and Equipment

A 40 W mercury germicidal lamp manufactured by Aqua Ultraviolet UV Sterilizer (Temecula, California) with a wavelength of 254 nm and constant photon output of 2.007mW/cm^2 was utilized to irradiate the microreactor. A syringe pump by Harvard Apparatus PHD 2000 Dual Syringe Pump (Holliston, Massachusetts) was programmed to inject the initial feed into the micro-reactor. HPLC tubing is fed from the syringe connectors to inlet port of the microreactor controlled by a split Y-junction connector and valves to control the flow and stream of liquid. The outlet port is connected to an Erlenmeyer flask with an arm that is attached to a High Efficiency Particulate Air Filter (HEPA) to prevent aerosolization of the sample.

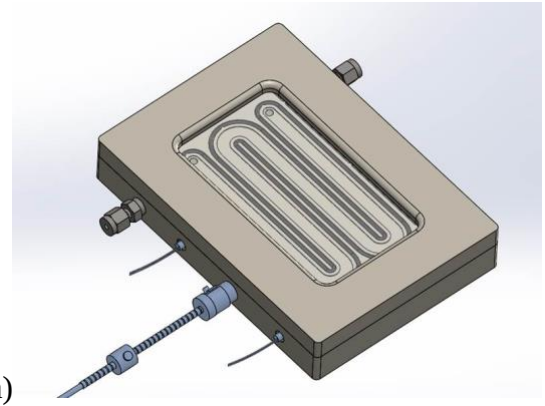
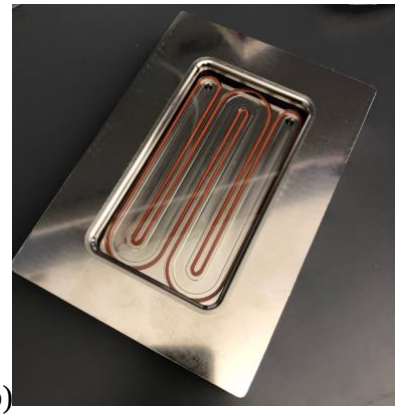
a)  b) 

Figure 8. A) Auto CAD representation of a prototype of the microscale UV water treatment unit. B) Actual prototype of microscale UV water treatment unit. The UV light source is not shown.

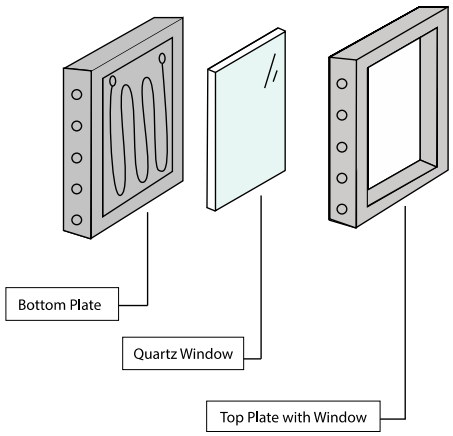


Figure 9. Expanded UV Microreactor Design, Left to Right: bottom plate, quartz window, top plate with window

The microreactor is comprised of a top and back plate of aluminum (Figures 6-9). The top plate consists of a window with a retainer for the quartz piece to be inserted on top of an O-ring. The back plate has a serpentine channel with a depth of 500 μm outlined with O-ring to seal the inner channel. Post-fabrication the reactor was electroless nickel plated to prevent corrosion by CG Industries (Albany, OR). Prior to the beginning and at the end of each experimental run the reactor was sterilized (Appendix A) and assembly aseptically and tightened with a torque wrench.

Reactor Assembly

The reactor is sterilized according to protocol (Appendix A) and is then aseptically assembled in a systematic fashion (Figure 10). The O-rings are inserted into the back and top plate followed by the quartz window and laid on top of each other. The screws are screwed in a star formation to equally distribute the pressure to the microreactor and are then tightened to the desired torque by a torque wrench (25 in-lb). The Y-junction is attached to the inlet and syringes

on the pump and the outlet is connected with tubing to the waste/collection vessel. Valves on fittings prior to the Y-junction control flow from syringes.

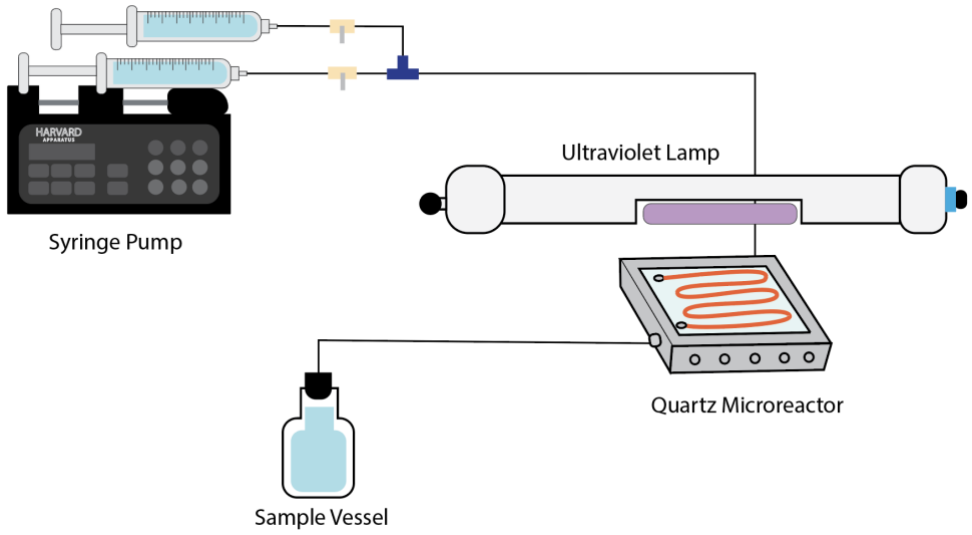


Figure 10. UV Reactor Flow System Set-Up

General Testing Procedure

The system is primed, and leak tested with a 60 mL Luer Lok Syringe (BD Medical, Franklin Lakes, NJ) filled with autoclaved deionized water to purge air bubbles from valves, Y-junction and the microreactor. The system is primed with autoclaved DI to charge the system with liquid. A sample containing known concentrations of the microorganism of interest is then added to the second line of the syringe to the system in a sterile 60 mL syringe by the syringe pump. The sample is run through the microreactor for a desired residence time within the microchannel that is determined by the volumetric flow rate of the syringe pump. The outlet tubing is moved from the waste vessel to the collection vessel after the required washout time, approximately 1 mL of the sample is collected and stored at 4°C for further processing.

Ultraviolet Quantification

An Avantes "AvaSpec-3648" UV-VIS spectra-photometer (Louisville, CO, USA) was used to quantify the flux of the ultraviolet light from the UV source. A constant flux of 2.007 mJ/cm^2 was found at 1-inch distance from the reactor. Total irradiance was measured prior to each experiment.

Enumeration of Cryptosporidium parvum

Sample Preparation

The standard operating protocol is provided in Appendix A. A desired concentration of *Cryptosporidium parvum* was diluted from a stock sample purchased from University of Arizona (UoA; School of Animal and Comparative Biomedical Sciences, *Cryptosporidium* Production Laboratory, Tucson, AZ). The sample is added to autoclaved deionized water and mixed with a spin bar to ensure adequate distribution within the liquid prior to uptake into a 60 mL syringe.

Sample Collection, Vital Dye Assay and Excystation

Approximately 1-2 mL of effluent samples from the microreactor were collected in 50 mL sterilized centrifuge tubes at desired residence times after a standardized washout time of the sample also calculated based upon the volumetric flow rate. 100 uL of the sample was added to 900 μL of acidified Hanks Balanced Salt Solution (HBSS) (pH 2.74, appendix A) and incubated for 1 hour at 37°C. Following incubation, the sample was rinsed, centrifuged and supernatant was aspirated three times with HBSS (pH 7.4) to remove residual acidified HBSS. The sample was then resuspended to 100 μL total volume with HBSS and 10 uL of 4',6-diamidino-2-

phenylindole (2mg/ml) (DAPI; MP Biochemical, Santa Ana, CA) and 10 μL of Propidium Iodide (1mg/mL) (PI; MP Biochemical, Santa Ana, CA) was added (manufacturer) and vortexed to completely mix. The sample was incubated for 2 hours at 37°C. Following incubation, the sample was rinsed by centrifugation with 100 μL of HBSS 3 times to remove residual dye. 5 μL of the sample was aliquoted to view within a Neubauer Hemocytometer (Bulldog Bio Inc., Portsmouth, NH). These samples were than assessed for oocysts viability by excystation. To the remainder of the sample, 200 μL of bovine bile solution (1% bovine bine in HBSS) and 50 μL of sodium bicarbonate solution (0.44% sodium hydrogen carbonate in distilled water) was added and incubated for 3 hours. Post incubation, the samples were washed thrice in HBSS and viewed under epifluorescence microscope.

Sample Quantification

Samples were viewed within 72 hours of processing under a fluorescence microscope (Leica DM2500) with a DAPI excitation filter (358-461 nm) and PI excitation filter (525-617 nm). Hemocytometers were divided into 4 sections and counting of cells was done systematically scrolling up and down with the DAPI filter selected first, followed by the PI filter prior to moving onto the next section to ensure accurate counts.

Enumeration of *Escherichia coli*

Sample Preparation

The standard operating protocol is provided in Appendix A. A stock sample of *Escherichia coli* (ATCC#25922) was utilized to grow a broth culture prior to processing. An inoculation loop with the stock is added to a conical flask with Luria Bertani Broth (LB;

Mumbai, IN). Flasks were incubated overnight at 37°C overnight for 18-24 hours. The broth was then placed into a sterile 60 mL syringe and is passed through the microreactor. 1 mL of the broth was aliquoted prior to quantify the starting concentration of the broth.

Sample Quantification

To evaluate the initial concentration of the broth and samples from the microreactor, a 10-fold serial dilution in phosphate buffered solution (PBS) was spread plated onto MacConkey agar and incubated at 37°C for 18-24 hours (Appendix A). Plates with colonies displaying dark pink with surrounding bile salt precipitate were counted as presumptive *E. coli*. Colonies were counted on dilution plates and the concentration was back calculated using equation 2.

$$\frac{(\#\ of\ colonies\ counted)\ (Dilution\ Factor)}{Volume\ Spread\ Plated} = CFU/mL$$

Equation 2. CFU/mL back calculation of *E. coli* concentration

Enumeration of *Bacteriophage MS2*

Stock preparation

The standard operating protocol is provided in Appendix A. A stock culture of *Bacteriophage MS2* was propagated by taking plates with growth and adding 3 mL of PBS. The top agar on these plates were scraped off and centrifuged at 1000 RPM for 25 minutes and aseptically filtered through a 0.22 μM syringe filter (VWR, Radnor, PA). The stock was then quantified by plating and equation 3 to determine concentration.

Sample preparation

Bacterial cultures of *E. coli* (ATCC#700891) host were streaked onto antibiotic supplemented 1.5% Tryptic Soy Agar (TSA) plates to obtain a pure isolate. This culture was then incubated over night at 37°C. To a conical flask, with ~25-30 mL of Tryptic Soy Broth (TSB; Hardy Diagnostics, Santa Maria, CA), a loopful of *E. coli* from frozen stock was inoculated to the flask. The broth was incubated overnight at 36°C or for 4-6 hours while shaking at 100-150 rpm. The broth was then serially diluted, with a minimum number of four dilutions.

Sample quantification

A double agar layer method was performed for a qualitative enumeration procedure of MS2 male-specific coliphage. 75 µL of host *E. coli* strain (ATCC#700891) was added to 0.7% Tryptic Soy Agar (TSA; Hardy Diagnostics, Santa Maria, CA) while molten, swirled to incorporate and then poured on top of the 1.5% hardened Tryptic Soy Agar and spiked with 100 µL of *Bacteriophage MS2*. A method blank was performed between each dilution to ensure there was no contamination. The plates were incubated overnight at 37°C and examined for circular lysis zones which indicate the presence or absence of coliphages via plaque forming units (PFU). The number of plaque forming units from the plated dilutions were calculated with Equation 3.

$$\frac{pfu}{mL} = \frac{(PFU_1 + PFU_2 \ldots PFU_n)}{(V_1 + V_2 \ldots V_{n)}}$$

Equation 3. PFU/mL Back calculation of *Bacteriophage MS2 concentration*. Where PFU = number of plaque forming units from all plates of all countable sample dilutions, V = volume of undiluted sample in all plates with countable plaques, n = number of usable counts

CHAPTER 7: RESULTS AND DISCUSSION

Overall Microreactor Results

The microreactor was evaluated for removal capabilities of *C. parvum*, *E. coli*, and *Bacteriophage MS2*. Reactor residence times were limited by the size of the syringe and the ability of the syringe pump. For all three pathogens the shortest working residence time was a volumetric flow rate of 65 mL/min or 1.57 seconds microreactor residence time. Deviations in collected data values can be attributed to many factors, but the most prevalent may be the addition of air bubbles to the reactor chamber over time when syringes were changed during a run. The addition of the bubbles changes the residence time within the reactor because the bubbles occupy the space that the liquid would travel through. This may alter the duration in which the microbes come into contact with the ultraviolet light.

For *Cryptosporidium* the longest residence time tested was 360 seconds yielding an average of 1.38 log removal. Interval times of 1.57s, 15s, 120s, were evaluated and yielded average log removal values of 0.17, 0.95, 1.33 oocysts/mL. For *E. coli* residence times of 1.57s, 5s, 8s, 15s, and 30s were evaluated and yielded an average log removal value of 2.18, 4.57, 7.48, 7.30, and 7.90 CFU/mL. *Bacteriophage MS2* was evaluated at 1.5s, 15s, 30s, 120s, 360s, residence times for respective log removal values of 0.37, 0.95, 2.78, 3.81, 5.02 PFU/mL (Figure 11) (Table 8).

Microreactor inactivation of *Cryptosporidium parvum*

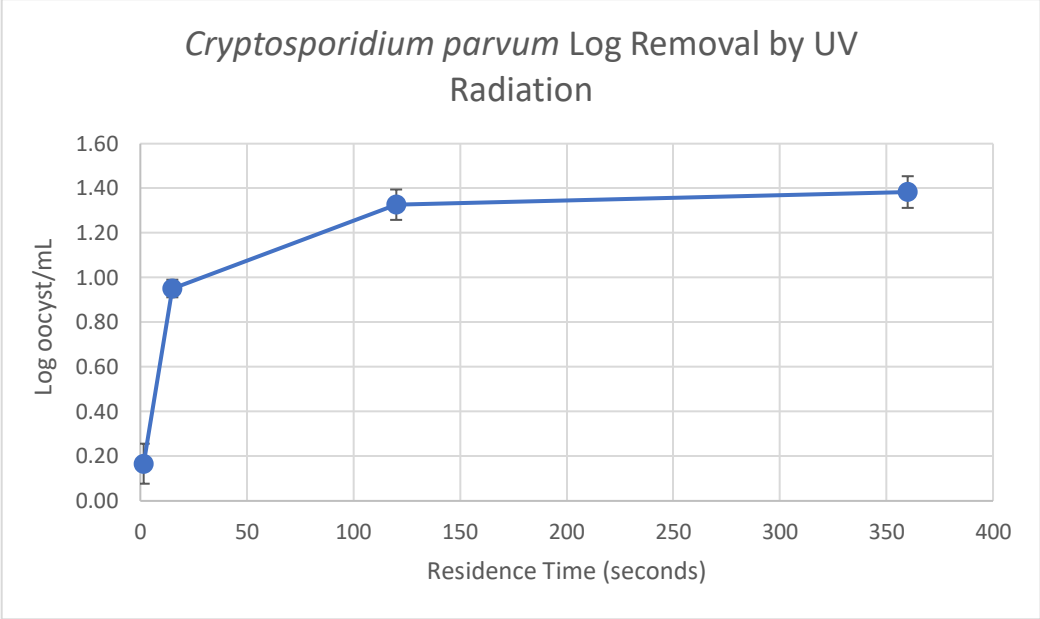


Figure 11. Log Removal by Ultraviolet Radiation of *Cryptosporidium parvum* at varying residence times.

Cryptosporidium was enumerated within the microreactor at 1.57s, 15s, 120s, and 360s and yielded respectively log removal values of 0.17, 0.95, 1.33 and 1.38 oocysts/mL (Figure 11, Table 6).

Residence Time	Trial 1	Trial 2	Trial 3	Average Log Removal	Standard Deviation
		Cryptosporidium parvum			
1.57	0.27	0.12	0.11	0.17	0.09
15	0.99	0.96	0.91	0.95	0.04
120	1.25	1.38	1.35	1.33	0.07
360	1.37	1.46	1.32	1.38	0.07

Table 6. Log Removal values for varying residence times of *C. parvum* ultraviolet exposure

We were unable to see adequate log-removal of *Cryptosporidium parvum*, which may be due to our enumeration methods and not the ability of the microreactor to inactivate the pathogen. The method in which we inactivate *Cryptosporidium* is molecular because the DNA is directly impaired by exposure to ultraviolet radiation. We experienced difficulty enumerating *Cryptosporidium parvum* post reactor treatment, specifically in visualizing cells that expressed PI staining. The difficulty in visualizing PI + cells may be because the use of viability stains is directly correlated to the condition of the cell wall, molecular damage alone is insufficient at visualizing "dead cells" post treatment. To remediate this, the oocyst cell walls must be physically damaged into order to allow the dead stain to come into contact with the DNA and indicate that a cell is dead.

An in-vitro excystation method was applied with the use of bovine bile and a sodium bicarbonate solution after the sample underwent dual incubation with vital dyes. The in-vitro excystation method provides an environment for the oocyst calcium cell wall to disintegrate due to its acidic pH and the cysts are free to leave or excyst. From here we are able to quantify the number of cysts present in the sample that have up taken the dead stain and can differentiate between the total number of cells that are alive or dead. However, it is important to note that in-vitro excystation methods have been known to overestimate viability of a sample (Campbell, 1992). It was still difficult to visualize PI + cells within the microscope due to the amount of interference in the sample. The sample viewed in the scope was clouded by empty shells and extracellular debris due to the cysts leaving their shells. With current funds, materials and methods of quantification it is not possible to quantify beyond 2 log-removals of *C. parvum*. A viable method for further quantification in the future that would reduce quantification time and

inaccuracy would be use of cell-culture methods or flow cytometry instead of vital dye assays. For future tests, a syringe pump able to process more than 60 mL volumes will be necessary to see larger than 2-log removal of the sample.

Microreactor inactivation of *Escherichia coli*

Rapid removal of *Escherichia coli* was achieved within the microreactor at multiple residence times. The mechanism of inactivation for *E. coli* with ultraviolet light is identical to *Cryptosporidium* in that the DNA is directly targeted and dimers are formed between mismatched nucleic acids. E. coli was exposed to UV-C light at 254 nm at 1.57s, 5s, 8s, 15s, and 30s at a distance of 1.25 inches from the light source. For samples exposed at 1.57, 5, and 8 residence times a respective 2.18, 4.57, and 7.48 Log CFU/mL were removed. At 15 seconds within the microreactor an average of 7.30 log CFU/mL) was found. Lastly, 7.90 log removal was achieved with exposure to the ultraviolet light at a 30-second residence time (Figure 12, Table 7). It was seen as the residence time in the microreactor decreased *E. coli* was more resilient to inactivation which is correlated to Kim et. al, 2013. There is sufficient evidence to conclude that inactivation of *E. coli* is a function of flux and increasing the contact time generates a higher level of *E. coli* inactivation. The increased exposure to ultraviolet light allows for the formation of additional photoproduct lesions within the DNA strand, the increase of dimers formed elicits more inactivated bacteria.

Residence Time	Trial 1	Trial 2	Trial 3	Average Log Removal	Standard Deviation
E. coli					
1.57	2.24	2.12	2.18	2.18	0.06
5	4.50	4.41	4.81	4.57	0.21
8	8.41	7.00	7.04	7.48	0.80
15	7.23	7.41	7.27	7.30	0.09

30	8.61	7.57	7.53	7.90	0.61

Table 7. Log Removal Values for varying residence times of *Escherichia coli* ultraviolet exposure

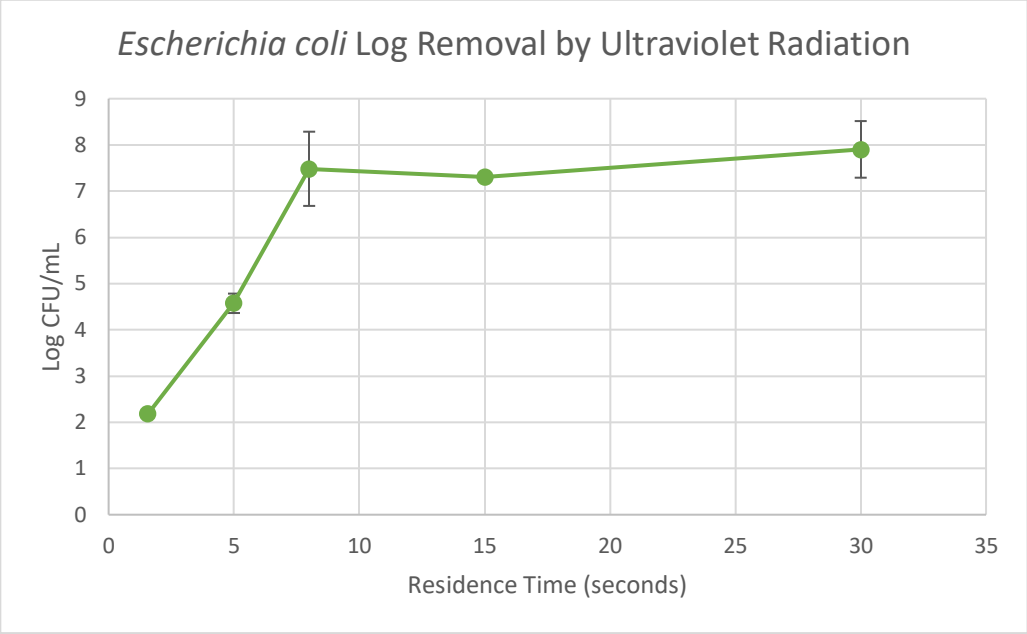


Figure 12. Log Removal vs. Residence time of *Escherichia coli* within the microreactor

Concentration of Flux and Turbidity

Preliminary experiments utilizing a smaller microreactor (Flow Cell Microreactor) with a low wattage lamp (8W) resulted in quantification complications with turbidity (Figure 13). When samples with concentration values greater than 10^6 CFU/mL were tested, UV light attenuated quickly within these samples due to their higher visual turbidity which yielded lower log removal values. This may be a problem in terms of competition with large-scale wastewater treatment systems as they are able to effectively tackle turbidity issues but could potentially be remediated with a filtration step in the pre-process.

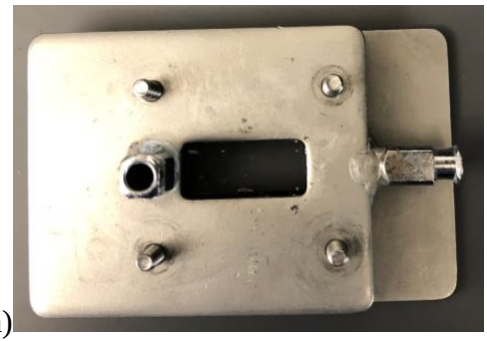
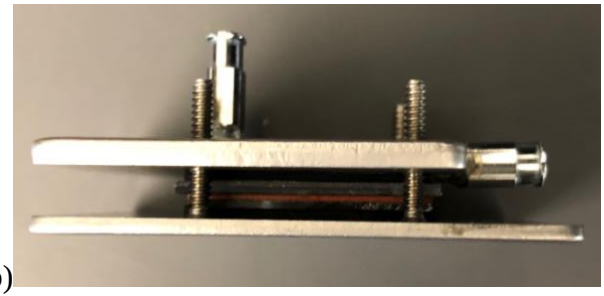
a)  b) 

Figure 13. Flow Cell Microreactor a) view from top of the microreactor b) side view of microreactor

The Flow Cell Microreactor was utilized in the beginning of the project for safety with *E. coli* and was evaluated at 15 and 30 seconds for removal. In the previous smaller set-up at 15- and 30-second residence times, log removal was approximately 4 and 6-log CFU/mL respectively. However, we were unable to quantify log removal values when turbidity of the sample grew too high. Because the sample was taken directly from a bacterial broth, the concentration could not be controlled and if the sample was not diluted, turbidity from the broth was high as well. When samples that were visually more turbid were processed, we were unable to quantify colony forming units due to the counts being too numerous to count. This data shows that turbidity of the sample or the concentration of the UV flux emitted from the light source inhibited the small reactor's ability to inactivate *E. coli*.

With the current UV microreactor set up, we encountered barriers with low log removals when using the same low power lamp as was used in the photoreactor. Testing with the 8W lamp at 30 seconds yielded approximately 3-log removal (CFU/mL). After replacing the UV source with a higher energy output lamp (40 W), while working with the same sample concentration, at a residence time of 30 seconds log removal was 7.90 log CFU/mL. From this data it was reasonable to conclude that turbidity does not play a role in inactivation kinetics of *E. coli* within this microreactor system when a higher flux of UV light is utilized.

Inactivation was found to be a function of distance, in which when distance increased a lower amount of log removal was observed. At 30 seconds, when distance was increased from 1-inch to 2-inches the average log removal was found to be too many to count. The reduction of inactivation with increasing distance from the UV source is widely accepted within literature and correlates with several studies performed (Liu, 2006) (Kim, 2013).

Microreactor inactivation of Bacteriophage MS2

The microreactor was extremely successful at inactivating *Bacteriophage MS2*. Residence times of 1.57s, 15s, 30s, 120s, 360s, and 480s were tested within the microreactor and yielded average log removal values of 0.37, 0.95, 2.78, 3.81, 5.02, and 6.96 PFU/mL respectively (Figure 14, Table 8).

Residence Time (s)	Trial 1	Trial 2	Trial 3	Average Log Removal (PFU/mL)	Standard Deviation
Bacteriophage MS2					
1.57	0.25	0.38	0.47	0.37	0.11
15	1.00	1.04	0.82	0.95	0.12
30	2.26	2.44	3.64	2.78	0.75
120	4.37	3.74	3.31	3.81	0.53
360	5.62	5.22	4.22	5.02	0.72
480	7.44	6.63	6.81	6.96	0.43

Table 8. Log Removal Values for varying residence times of *Bacteriophage MS2* ultraviolet exposure

According to literature values, inactivation of *Bacteriophage MS2* via ultraviolet light requires substantially higher flux and contact times than both *E. coli* and *C. parvum*. However, we found that successful inactivation of *Bacteriophage MS2* with our given set-up. With our

longest residence time of 480 seconds (8 minutes) yielding an average of 6.96 log PFU/mL removed.

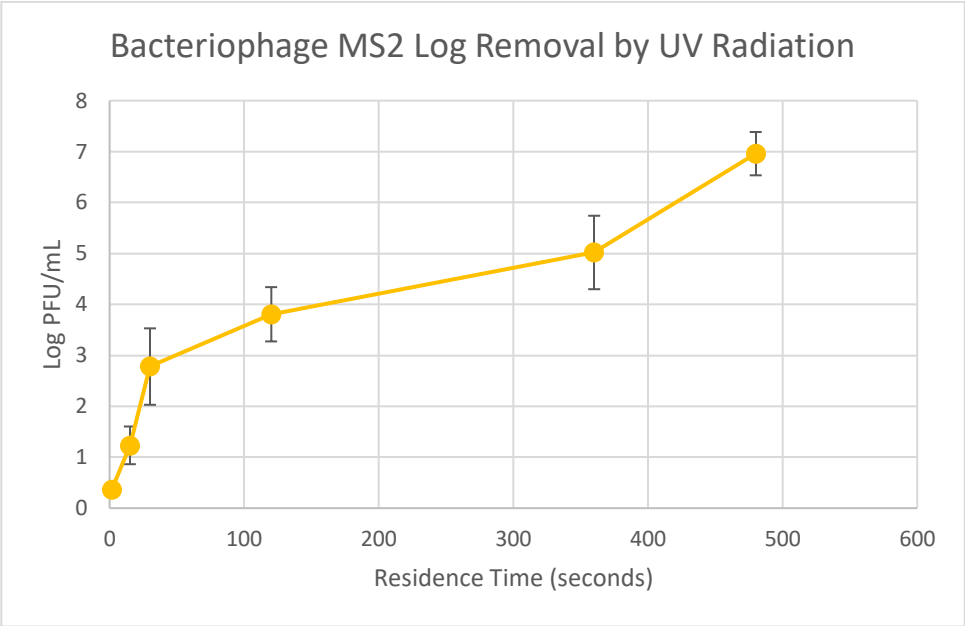


Figure 14. Log Removal vs. Residence Time of *Bacteriophage MS2* at varying residence times

The inactivation of *Bacteriophage MS2* and *Escherichia coli* from exposure to ultraviolet light were expected from our results. However, our data for *Cryptosporidium* does not generate the same level of log-removal values as the other microbes. We postulate that this is not because the microreactor is unable to inactivate *Cryptosporidium parvum* but because our enumeration methods are not adequate. The EPA's Method 1623 on *Cryptosporidium* enumeration states the use of a phase contrast microscope and 200-400X magnification on the fluorescence microscope however the maximum magnification possible on ours was 50X (EPA, 2005). The inability to accurately determine PI+ samples from microscopy techniques pre and post excystation may have led to an underestimate in viability counts of *C. parvum*.

According to the Ultraviolet Disinfection Guidance Manual for LT2ESWTR, posted by the EPA, when a technology treatment system undergoes validation, a more robust pathogen like *Bacteriophage MS2* may be utilized to qualify for a more difficult to enumerate pathogen like *C. parvum* (Beck, 2016), (EPA, 2006). The amount of inactivation is determined from a validation factor that is performed once the system is ready for distribution according to EPA guidelines (EPA, 2006). 6-log removal of *Bacteriophage MS2* qualifies a system for "3-to-4-logs" of removal for viruses (EPA, 2006). From our data, at our longest residence time (480s/8 min) an average of 6.96 log PFU/ mL was removed of *Bacteriophage MS2* which indicates that our reactor should be also able to inactivate *C. parvum* as well.

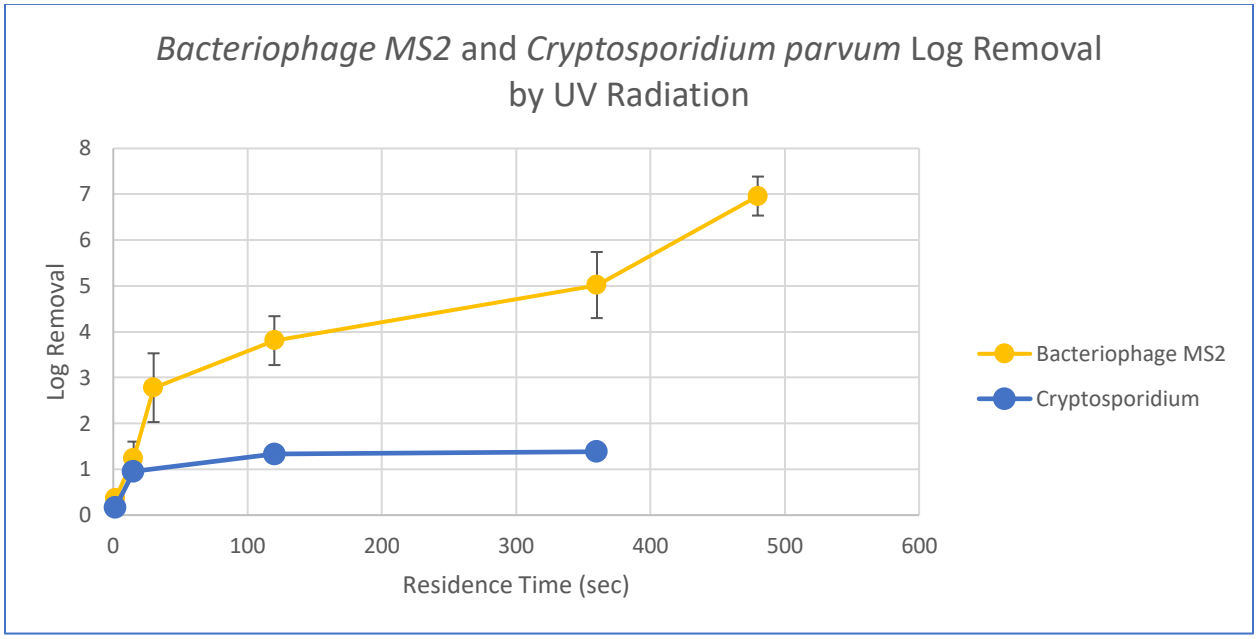


Figure 15. Comparison of *Bacteriophage MS2* and *Cryptosporidium* Log Removal by Ultraviolet Radiation

From the data, the curves are similar and both *C. parvum* and *Bacteriophage* have similar initial log-removal values at 1.57s with a respective 0.17 oocyst/mL and 0.37 PFU/mL (Figure

15). The similarities continue at 15s within the reactor with 0.95 oocyst/mL and PFU/mL but differentiate at the 120 second residence time with values of 1.33 oocyst/mL and 3.81 PFU/mL which is interesting because *Bacteriophage MS2* is more robust and should be more difficult to inactivate than *C. parvum*. Enumeration of Bacteriophage MS2 is significantly easier as it is culture-based standardized double agar overlay method whereas *C. parvum* enumeration is not standardized (even though there are EPA protocols) and has more steps that can contribute to human error. There are several aspiration steps in *C. parvum* enumeration that may reduce the number of viable cells upon microscope counts. With *C. parvum* because it is a larger microbe, the distribution of the sample within the syringe to the reactor chamber is another point of contention because the sample is not well mixed. The oocysts may settle to the bottom of the syringe and when the sample passes through the reactor chamber a representative sample is not collected.

Comparison of Our System to Standard Treatment Systems

According to the LTSWTR2 UV disinfection manual, the typically flux of UV applied for inactivation of *Cryptosporidium* and virus are indicated below in Table 9. Within the manual, a contact time for each dosage of UV was not listed.

Table 9. UV Dose Requirements – mJ/cm^2 (adapted from EPA UV Disinfection guide (EPA, 2006)

Target Pathogens	**Log Inactivation**							
	0.5	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**
Cryptosporidium	1.6	2.5	3.9	5.8	8.5	12	15	22
Virus	39	58	79	100	121	143	163	186

A comparison of our UV flux values to those of the EPA demonstrate a lower flux value achieving equal if not more log removal for each pathogen. For *C. parvum*, our flux of 2.007

mJ/cm^2 yielded 1.3 log oocyst/mL removed at a 2 minute residence time. The EPA values for 1 log require 2.5 mJ/cm^2 and for 1.5 log, 3.9 mJ/cm^2 which is quite close to what we have accomplished at a lower flux. For *Bacteriophage MS2*, we have achieved over 4.0 Log PFU/mL with 2.007 mJ/cm^2 where in traditional treatment, 4.0 Log removal UV dose requirements from the EPA state that 186 mJ/cm^2 are required. Our system has achieved 2 magnitudes of order lower at 4.0 Logs removed than what is utilized in traditional treatment at a residence time of 6 minutes. We suspect these values are higher due to a better design approach that effectively utilizes the ultraviolet light from the bulb with little attenuation. It is also possible that the recommended dosage for UV is significantly higher than our values because of the broad spectrum of viruses that must be treated in drinking water systems. Adenovirus is a notably difficult virus to inactivate and requires polychromatic light, while our microbes of interest require monochromatic light (one output wavelength of 254 nm). The larger range in numbers may be set to compensate for a wide range of viruses (Chatterley, 2010).

Compliance with WHO International Scheme of Household Water Treatment Technologies

Table 4. World Health Organization (WHO) International Scheme to Evaluate Household Water Treatment Technologies

Performance Classification	Bacteria (log reduction required)	Viruses (log reduction required)	Protozoa (log reduction required)	Interpretation
3	4-log or more	5-log or more	4-log or more	Very high pathogen removal
2	2-log or more	3 log or more	2-log or more	High pathogen removal
1	Meets at least level 2 performance classification for 2 classes of pathogens			Targeted Protection

With WHO standards we comply with a performance 2 classification, meeting all 3 parameters of log removal for bacteria, virus, and protozoan characterizing our system as capable of high pathogen removal (Table 4). Depending on the validation factor for protozoan we could also potentially achieve a performance 3 classification, in which we have exceed the 4-log reduction for bacteria, and the 5-log reduction for virus. Potentially after a new design and optimization of the microreactor a solid performance 3 classification for very high pathogen removal will be possible.

CHAPTER 8: CONCLUSIONS AND RECOMMENDATIONS

Access to modular point of use technology in drinking water disinfection is a necessity. The efficacy of a microscale fluidic reactor was evaluated for treatment capabilities amongst 3 regulatory categories of microorganisms: bacteria, protozoan, and virus. The microreactor tested was 0.5 microns deep with a serpentine channel and quartz window for optimal exposure to ultraviolet light. A constant intensity of ultraviolet light through the quartz window of the microreactor irradiated the microorganisms. Different volumetric flow rates controlled the exposure time to ultraviolet light of each microorganism and generated varying levels of log removal post quantification. Reactor residence times were evaluated from 1.57 seconds to 6 minutes and were limited by both the volume of the syringe and the capabilities of the syringe pump.

The microreactor was successful at inactivating all three classes of pathogens. This work confirms current literature statements that inactivation of these pathogens are directly correlated to the exposure time to ultraviolet radiation. *E. coli* and *Bacteriophage MS2* were inactivated as expected however the expected trend of *C. parvum* was not observed. We postulate this can be

due to improper enumeration methods/lack of access to adequate enumeration equipment. We do not believe that the microreactor is incapable at inactivating *Cryptosporidium parvum* to the same level as *Bacteriophage MS2* as *MS2* is often used as a viral indicator in standard water treatment to qualify for *C. parvum* removal. More research is needed to evaluate the microreactor's true efficacy at removing *C. parvum*.

Experimental and Microreactor Recommendations

Incorporation of ultraviolet LED lights should be implemented to enhance the accessibility of the reactor in future commercialization aspects. Redesign of the reactor to include a lower channel depth should also be implemented to reduce potential effects from attenuation. The reduction of channel depth may also affect the effectiveness of the microreactor in inactivating *Cryptosporidium parvum*; potentially yielding higher rates of inactivation. To further quantify inactivation of *Cryptosporidium parvum* to the same standard as *Bacteriophage MS2* and *Escherichia coli*, infectivity studies with cell culture must be evaluated as a quality control measure to validate the device for inactivation. In regard to *Escherichia coli*, samples with higher levels of turbidity should also be investigated for quality control measures.

In addition to incorporation of UV LEDs, quantification of the total average flux within the reactor should be performed with a photon flux detector. Because we only measured the flux in the reactor that was directly parallel to the light, we are underestimating the total average flux. There are other incidences of light from the bulb that may pan out further than directly parallel on the microreactor.

Future Work

Integration of advanced oxidation processes via photocatalysis with titanium dioxide should be investigated to examine rates of inactivation for all three classes of microbial pathogens. Once microbiological components are evaluated, different emerging contaminants should be evaluated for degradation within this microreactor. Compounds like pharmaceutical drugs and waste, endocrine disruptors, birth control, personal care byproducts and other common compounds often found inhibiting water quality should be investigated in separate and combined systems.

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CHAPTER 10: APPENDICES

A. Standard Operating Protocols

B. Microreactor Flow Rates

A.1 Protocol: UV Microreactor Flow System Standard Operating Procedure
Purpose: Standard Operating Procedure (SOP) for parts and assembly, and operation guidance

Materials:
II.

Item	Quantity	Company	Part Number
PEEK Tubing Natural 1/16'' OD x .040" ID x5ft	1	IDEX	1538
Flangeless Nut PEEK, 1/ 4-28 Flat-Bottom, for 1/16" OD (Natural)	2	IDEX	P-230
Flangeless Ferrule Tefzel (ETFE), 1/ 4-28 Flat-Bottom, for 1/16" OD Blue-10 Pack	1	IDEX	P-200X
Female LuerTight FItting for 1/16" OD	2	IDEX	P-835
Male LuerTight Fitting System for 1/16" OD	3	IDEX	P-836
Ultraviolet Light System	1		
Kimwipes	1	Chemstores	
Ethanol 70% (made in house)		Chemstores	
Plastic Disposable Syringes (60mL)	1 per 4 samples	BD Medical	
UV Goggles	4	UVEX/VWR	
Teflon Tape			

Part Number	Description
Aluminum Bottom Plate	MBR3-2001
Aluminum Top Plate	MBR3-2002
Aluminum Top plate Window	MBR3-2003
Aluminum Top plate Window Retainer	MBR3-2004
High Temperature silicone O-ring for Top Plate	1283N214
O-ring High Temperature silicone Inner seal for bottom plate	1169N301, 12.3in ID

Table of Contents

1. Safety

a. Ensure everyone working with the microreactor is wearing:
b. Ultraviolet Light eye protection
c. Gloves
d. Sleeves are rolled down to prevent skin exposure
e. Closed toe shoes and long pants
    i. – face mask is optional*

2. Overall System Schematic

IMAGE OF FLOW SCHEMATIC

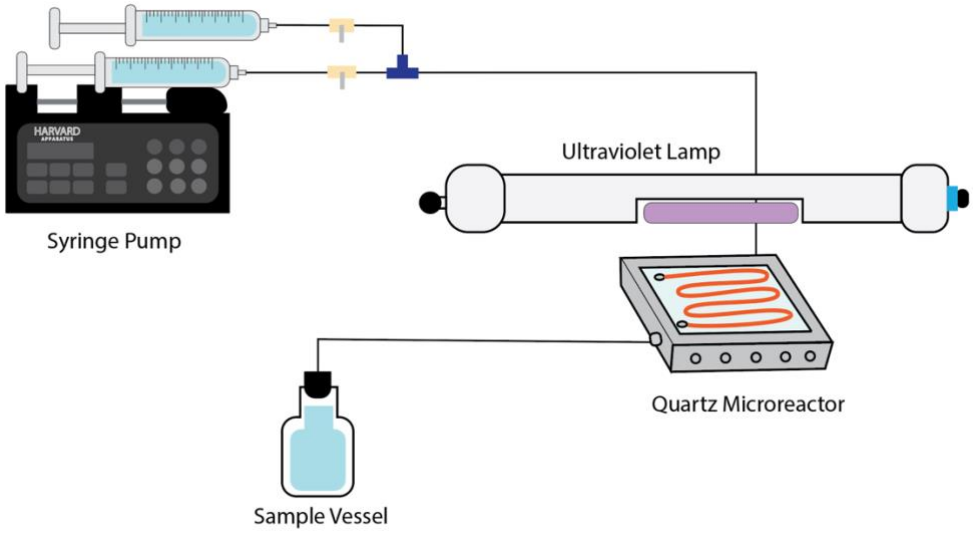
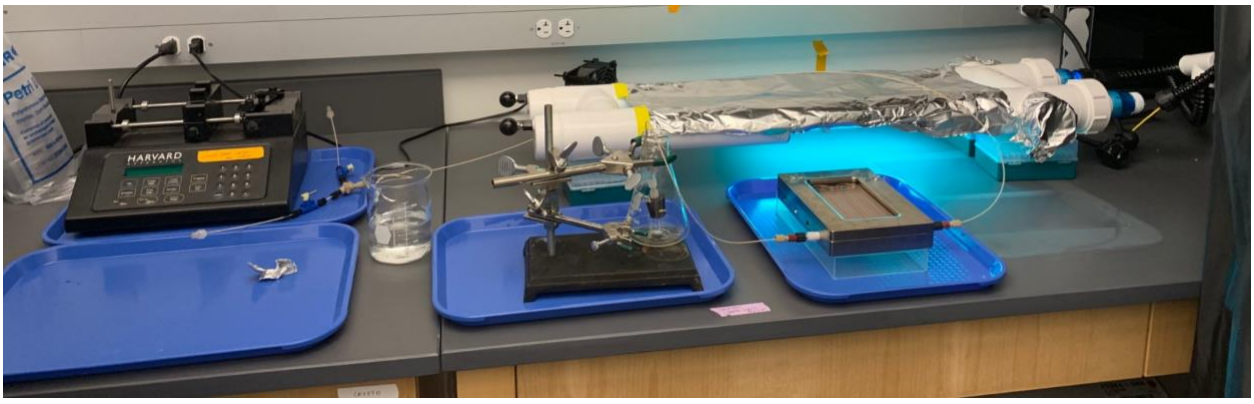


IMAGE OF ACTUAL SYSTEM



I. Reactor Assembly

1. Sterilize the reactor
    a. Refer to **Section IV. Sterilization**
2. Clean Quartz Window
    a. Scrub quartz piece with a brush using soap and water
    b. Spray ethanol onto quartz piece rinse with DI
    c. Lay down a clean towel and use a kimwipe to dry the quartz piece to remove dust/hairs

d. Keep stored in "Quartz UV Box" when not used

3. Prop reactor up with bottom plate first
    a. Insert the O-rings on both plates aseptically with gloves from the biosafety cabinet
        i. Top plate: insert orange O-rings
        ii. Bottom plate: insert black O-ring and then place quartz piece on top (cracked side facedown into top plate)
            1. Buna-N o-ring (Black et al.) max temp 121C
    b. Flip bottom plate on top of top plate (plate with window) and chec to make sure o-rings have not dislodged before screwing
    c. Align plates and put screws into place
    d. Begin to loosely screw in "star" formation with regular screwdriver
    e. With a torque wrench, brace the reactor and tighten screws with desired torque in star formation
        i. 10 lb x force / in = determined torque applied

II. Prepping System for Run

1) Safety
    a) Ensure every operator is wearing UV glasses
    b) Ensure the curtain is closed

2) Leak Test
    a) Attach DI syringe to pump
    b) Attach fluidic connectors to reactor and syringe port tightly
    c) Make sure correct valves are open
    d) Place outlet tubing into waste vessel
    e) Turn on pump to flush system and check for leaks (12 mL/min is sufficient)
    f) If leaks are still present, troubleshoot fittings/re-tighten screws if necessary and flush system again
    g) If air bubbles appear attempt to push them out, if it does not work, breakdown reactor and clean according to Sterilization Protocol and reassemble

3) Reactor Set-Up
    a) Wear lab coat, UV goggles and all other appropriate PPE
    b) Close the curtain
    c) Wrap the sample vessel with foil to protect from UV exposure and have ready for collection
    d) Use the excel file to calculate necessary flow rate for desired reactor residence time
    e) Set the reactor, sample vessel and waste vessel on to the tray

f) Carefully take DI syringe off the pump as water may leak out and place on tray and close the valve
g) To the second line load a sample syringe with sample inoculation and connect syringe to tubing ports on the pump and open the valve
h) Place outlet tubing into waste vessel
i) Turn fan on to cool reactor

4) Priming the system
   a) Flow sample solution to remove air bubbles with pump
   b) Note: Higher flow rates (~5ml/min) can be used during this stage

III. Running the Reactor

***Everytime the flow-system box is open, UV goggles must be worn*

1) **Sample collection**
   a) Place the microreactor on the box and place underneath UV light so that at least one channel is directly exposed to the light
   b) Connect the outlet tubing to the waste vessel
   c) Close the flow-system box
   d) Turn the pump on and allow for a washout period of the sample for 3 residence times at the desired flow rate to allow the reactor to acclimate to bulb and flush the system (using DI syringe till 13.66 mL when using a 60 mL syringe)
   e) After washout collect the sample
   f) Start a timer for the desired collection time
   g) After the time has passed, stop the pump to conserve sample
   h) Transfer the outlet to the waste vessel and remove the sample vessel
      i) *If running a second sample with the same syringe, place second sample vessel in place of original
2) **Reloading the Syringe**
   a) Stop the pump
   b) Connect the new syringe to an open line (preferably the line that was originally DI water to prevent contamination)
   c) Set pump to desired value
   d) Remove the old syringe (depending on which pump, use the switch)
   e) Start the pump
3) **Stopping the reactor**
   a) Pause the pump to stop sample flow through the system
   b) Slide out sample tray from underneath light,
   c) Remove sample vessel and replace with waste vessel
   d) Turn off pump

IV. Sterilizing/Cleaning the Reactor

For C. parvum

1. Set up water bath by turning on hot plate to 200°C, place metal pot on top of hot plate, and add water
2. Load syringe with ethanol solution (70%) and flush system for 3 residence times at 10 ml/min
3. Transfer flow-system into secondary containment unit and begin disassembly
    a. Components for sterilization: fittings, connectors, tubing, reactor (window, bolts, o-rings), collection vessels
    b. Syringes will go directly into biohazard bin
    c. Quartz window must be sterilized separately (same procedure)
    d. Autoclave these items post-waterbath:
        i. All secondary containment units (biosafety cabinet transfer unit, flow-system tray unit, pump unit)
4. Carefully place components into hot water bath and sterlize for 10 minutes at 200 C temperature using tongs.
    a. Do not overload the capacity of the hot water bath
5. Once sterilization time has passed, remove the components individually with tongs
6. Wash with soap and water scrub the reactor and the quartz plate
7. Rinse with DI water and then spray with 70% ethanol and rinse with DI
8. Place sterile disassembled reactor into a sterile containment unit for next use.
9. Place tongs, tools, and other non-flow system componenets in hot water bath for 10 mintues at 100C temperure.
10. Turn off the hot plate.
11. Wait for water to cool prior to disposing into debris-free sink.
    a. If time constraints apply, add ice to hot water and then dump.

For Bacteriophage MS2 and Escheirichia coli

1. Load syringe with ethanol solution (70%) and flush system for 3 residence times at 10 ml/min
2. Disassemble reactor in secondary containment unit
3. Wash with soap and water scrub the reactor and the quartz plate
4. Rinse with DI water and then spray with 70% ethanol and rinse with DI

.

Figure X. UV light bulb ballast

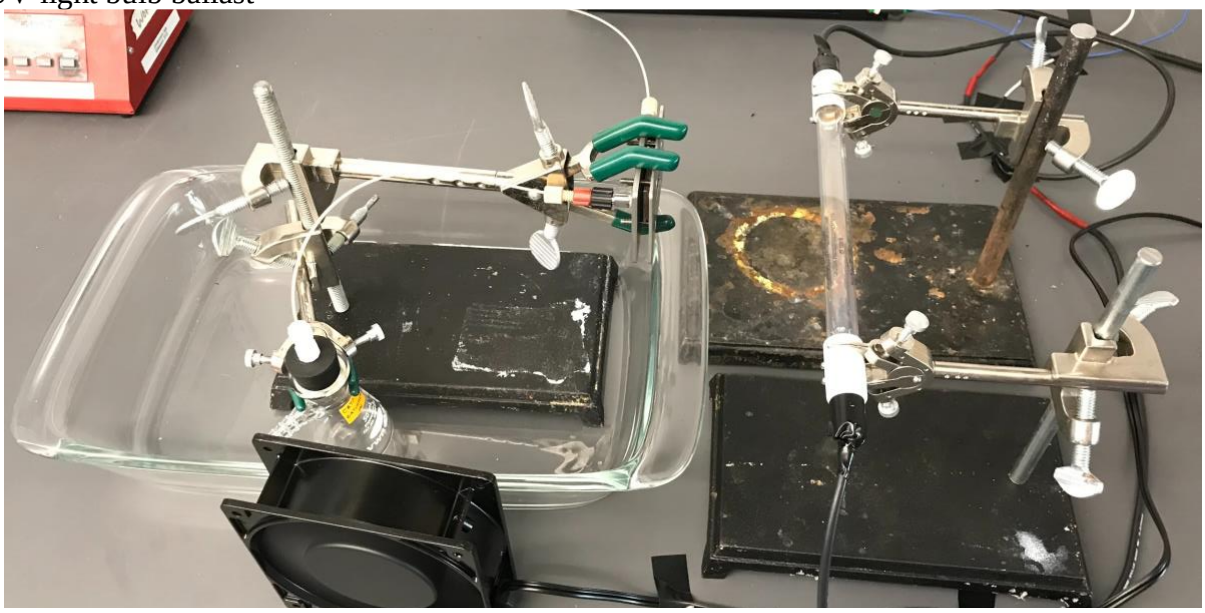


Figure X. UV Test Loop without UV shield box

A2. Bacteria Enumeration by Spread Plate Method

1. **Purpose**
   This protocol describes the process to determine the concentration of colony forming units from an effluent sample.

2. **Materials**
   E. coli (ATCC 25922)
   MacConkey Agar with Mug
   Phosphate Buffered Solution (PBS)
   Deionized water
   Ethanol (70%)

3. **Reagent Preparation**
   a. MacConkey Agar with Mug
      i. 49.53g (or specified amount on label) of agar powder
      ii. 1000 mL Deionized Water

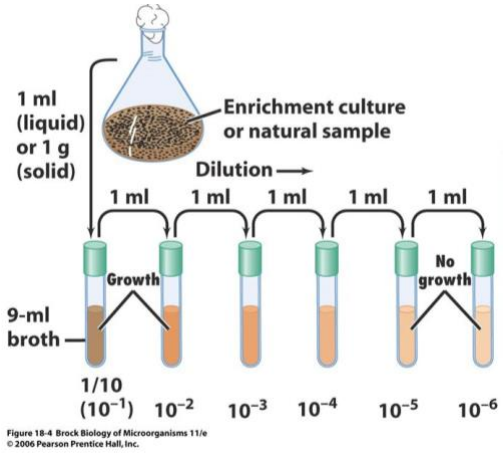
      Add materials to 1 L media bottle, shake to incorporate, open a quarter-turn and autoclave for 15 minutes at 121°C.

   b. Phosphate Buffered Solution (10x solution)
      i. 80g Sodium chloride
      ii. 2g Potassium chloride
      iii. 14.4g Disodium phosphate
      iv. 2.4g Monopotassium phosphate

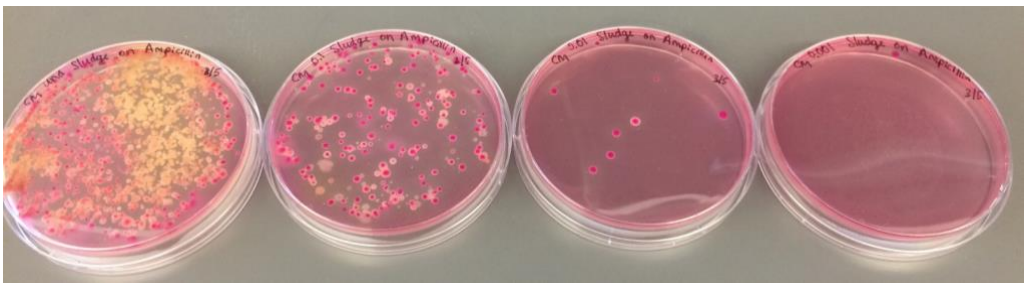
      Add materials to 1 L media bottle, shake to incorporate, open a quarter-turn and autoclave for 15 minutes at 121°C.

4. **Procedures**
   a. Preparation of MacConkey Plates
      i. Allow media bottle to cool until ~50-60°C or hand-hot temperature
      ii. Sterilize the countertop with ethanol and lay out plates
      iii. Pour plate evenly till about 1/3 of the plate is full, swirl intermittently to prevent clumping
      iv. Allow plates to cool and tape together (18 a stack) and store in the fridge at 4°C for 2 weeks.

   b. Dilutions
      i. Sterilize the countertop by wiping down with ethanol (70%)
      ii. Add 9 mL of PBS to test tubes to create appropriate dilutions for your sample (typically D1-D9)
      iii. Add 1 mL of sample to the first test tube (D1) to 9 mL of PBS and pipette air to mix thoroughly (~10 times)
      iv. Create a dilution series by adding 1 mL of the well mixed D1 to the next tube. Repeat for all test tubes.



c. Spread Plating
   i. Sterilize countertop by wiping down with ethanol (70%)
   ii. Prepare agar plates by labeling appropriately: dilution series, initials, and date
   iii. Plate the necessary dilutions (target is 25-250 colonies on the plate)
   iv. Mix the dilution tubes with a 1mL pipettor and take 75 uL of well mixed solution to the corresponding plate.
   v. Ethanol-flame sterilize the spreader and spread the solution with the glass/metal spreader in a forward and backward motion while rotating the plate
   vi. Allow the plates to dry and incubate the plates inverted for 24 hours at 35°C
   vii. Count colonies the following day



Example dilution series

d. CFU/mL Back calculation
   i. To calculate CFU/mL of the original sample, perform the following calculation:

$$\frac{\text{CFU}}{\text{mL}} = \frac{\text{\# colonies counted}}{(\text{Dilution Factor}) \times (\text{Volume Plated})}$$

5. *E. coli* Reactor Procedure

Materials

Luria Broth
60 mL Syringes
50 mL Centrifuge Tube
Test tubes
E. coli Stock (ATCC 25922)
MacConkey Agar Plates
Microcentrifuge Tubes
PBS Solution

a. Sample Preparation
    i. Create a broth of 25922 to run through the microreactor
        1. Make 60 mL of Luria Broth according to manufacturer's instructions
        2. Add 60 mL to an Erlenmeyer culture flask
        3. Aseptically inoculate 25922 from frozen stock by flame sterilizing the inoculation loop and then dipping it into the stock and into the broth
        4. Shake the loop into the broth to disperse the bacteria
        5. Incubate the broth for 18 hours at 35°C
        6. Take 1 mL of the broth to quantify broth concentration by serial dilution and spread plating
        7. Uptake remaining volume into syringe and load into microreactor for testing

b. Sample Collection
    i. Upon washout time (3 residence times) collect at least 100 uL of sample into 50 mL centrifuge tube

c. Enumeration of Samples
    i. Vortex collected sample
    ii. Serially dilute sample and plate all dilutions D0-DX in duplicates
    iii. Count plates and back calculate plate concentration
    iv. Find log removal by subtracting plate concentration from initial broth concentration

A3. Bacteriophage MS2 Enumeration by Double Agar Overlay Method

6. Purpose

This protocol describes the process to determine the concentration of plaque forming units from an effluent sample.

7. Materials

E. coli Host (ATCC 700891)
Ampicillin sodium salt Sigma A9518 or equivalent
Phosphate Buffered Solution (PBS)
Streptomycin sulfate Sigma S6501 or equivalent
Tryptic Soy Broth (TSB) DIFCO 0370-15-5 or equivalent
Bottom agar: 1.5% Tryptic Soy Agar (TSA)
Top agar: 0.75% TSA
11 X 100 mm glass tubes (needs to hold 10 mL each)
Ethanol (70% or greater)
Microcentrifuge Tubes
0.22 uM pore-size membrane syringe filter assembly (syringe filter + syringe)

8. Reagent Preparation

a. Stock Ampicillin/Streptomycin Solution
   i. 0.15g ampicillin sodium salt
   ii. 0.15g streptomycin sulfate
   iii. 100 mL DI water

   Add materials to 250 mL media bottle, shake to incorporate, decant into a beaker and uptake liquid with syringe. Attach syringe filter, and filter liquid into new sterile 50 mL centrifuge tube.

b. Tryptic Soy Broth (TSB)
   i. 30g
   ii. 1 L DI water

   Add materials to 1 L media bottle, shake to incorporate, open a quarter-turn and autoclave for 15 minutes at 121°C.

c. Tryptic Soy Broth with Antibiotics
   i. 30g
   ii. 1 L DI water

   Add materials to 1 L media bottle, shake to incorporate, open a quarter-turn and autoclave for 15 minutes at 121°C.
   iii. Once agar has cooled to 52°C add 10 mL of stock ampicillin/streptomycin solution to 1L of autoclaved TSB.

   Antibiotics must be added to the media after it has been autoclaved and cooled. Note: Antibiotics are sensitive to heat.

d. 1.5% Tryptic Soy Agar (TSA)

To be used as the bottom layer of plates for the double agar overlay method.

i. Prepare TSB and add 15g of regular agar powder per L of TSB
ii. Add to media bottle, shake to incorporate, open a quarter-turn and autoclave for 15 minutes at 121°C.
iii. Mix molten medium well to ensure even distribution and allow to cool to 52°C, invert the cooling agar intermittently to avoid solidifying.
iv. Once agar has cooled to 52°C add 10 mL of stock ampicillin/streptomycin solution to 1L of autoclaved TSB
v. Aseptically pour plates enough till the bottom is covered and store inverted at 4°C for 2 weeks.

Antibiotics must be added to the media after it has been autoclaved and cooled.
Note: Antibiotics are sensitive to heat.

e. 0.7% Tryptic Soy Agar (TSA)

To be used as the top layer agar ("soft" agar) for the double agar overlay method.

i. Prepare TSB and add 7g of regular agar powder per L of TSB
ii. Add to media bottle, shake to incorporate, open a quarter-turn and autoclave for 15 minutes at 121°C.
iii. Mix molten medium well to ensure even distribution and allow to cool to 52°C, invert the cooling agar intermittently to avoid solidifying.
iv. Once agar has cooled to 52°C add 10 mL of stock ampicillin/streptomycin solution to 1L of autoclaved TSB
v. Pre-warm 50 mL culture tubes in hot water bath (leave space for 0.7% TSA agar bottle – if not all is used)
vi. Dispense 8 mL per 50 mL culture tube with a 10 mL pipettor

9. Procedures

a. Preparation of Bacteriophage Stock

To be made when no bacteriophage stock is left.

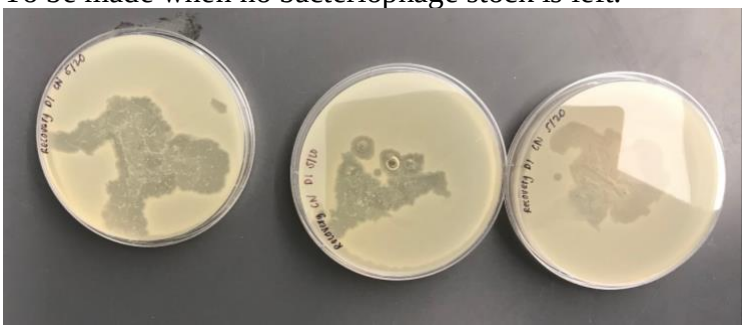


Figure X. Example of ideal plates for Bacteriophage Sample Recovery (D1-D3)

i. Sample must be propagated (made)
    1. Follow Bacteriophage Enumeration Protocol
    2. Plate D1-D3 dilutions for 24 hours at 37°C
    3. Take the plate directly from the incubator and add 3 mL of PBS to the plate
    4. Use a glass spreader to scrape off the top agar

a. It will be very easy because the agar is warm, not much scraping is needed

5. Use a 10 mL pipette tip to uptake the liquid and transfer the agar/liquid mixture to a 50 mL centrifuge tube
    a. If the agar is chunky, disrupt agar by gently pipetting up and down
6. Centrifuge at 1000 rpm for 25 minutes to sediment cellular debris and agar
7. Save and aliquot the supernatant into a new 50 mL centrifuge tube
8. Set up a vacuum filtration unit with a Whatman 1.5 uM Glass Microfiber filter and filter aseptically
9. Use a 10 mL Pipettor to uptake the filtered liquid and transfer to a new 50 mL centrifuge tube
10. Pour liquid into a sterile beaker and uptake into a sterile syringe
11. Attach the 0.22 uM syringe filter to the tip and filter
    a. This is difficult and takes time (~1.5 hours per 4 mL of liquid), multiple syringe filters can be used as well

ii. Sample is already made and taken from frozen stock
1. Sample will be in freezer in 50 mL centrifuge tube
2. Defrost and take as much as needed

b. Preparation of *E. coli* Host Stock (ATCC 700891)
i. Take a 125 mL flask and add 25-30 mL of TSB with ampicillin/streptomycin antibiotics
ii. Inoculate the flask with a loopful of *E. coli* from the frozen stock culture of (ATCC 700891)
iii. Place a sterile cap in the shaker flask, label and incubate at 37°C for 24 hours. Cultures should be visibly turbid to indicate log growth.
iv. Chill on wet ice or at 4°C until ready for use.

c. Enumeration of MS2 Bacteriophage (Double Agar Layer Method)
i. Preparing Dilutions
1. Sterilize countertop by wiping down with ethanol (70%)
2. Create dilutions of stock MS2 Bacteriophage sample in microcentrifuge tubes and TSB without antibiotics as the diluent
    a. Typically D1-D9, MS2 Stock concentrations are high
3. Take 100 uL of stock for dilution and add aseptically to 900 uL of TSB, repeat this serial dilution for the desired number of dilutions.
4. Vortex each tube on “6” to avoid disturbing the bacteriophage but to ensure it is fully incorporated prior to transferring to the next dilution.

ii. Agar Preparation

1. Autoclave the 0.7% top agar solution and leave in the hot water bath at 52°C until ready for use with 50 mL culture tubes preheating
2. Disinfect workplace with ethanol solution and prepare 1.5% TSA bottom agar plates and label with appropriate information (e.i., dilution factor, name, date, time)

iii. Enumeration Preparation
1. Disinfect workplace with ethanol solution
2. Dispense 8 mL of 0.7% top agar into preheated 50 mL culture tubes and keep in hot water bath
3. Work with one tube at a time, inoculate with 75 uL of *E. coli* culture (700891), swirl for 3 seconds to mix and pour the contents of the tube onto the top agar plate
4. Wait 10 seconds for the soft agar to harden and then add 100 uL of phage at the corresponding dilution to the plate in a spotting fashion
    a. Work aseptically by keeping the lid hovering over the plate to prevent aerosolization and contamination of the phage
    b. Run duplicates
5. Sterilize and run a method blank between each dilution series
6. To prepare a method blank inoculate the top agar tube with 75 uL of 700891 and then add 100 uL of TSB diluent without antibiotics into method blank plate
7. Wait for top agar plates to harden, invert, and incubate for 16 to 24 hours at 36°C.

iv. Enumeration
1. Method blank should show no growth, if growth is shown, new TSB and dilutions should be created
2. Count the number of plaque forming units on each plate
    a. Circular zones should be visible in lawn of host bacteria typically 1 to 10 mm in diameter
    b. Sum the number of PFU from all dilutions that are countable
    c. Sum the undiluted sample volumes used to inoculate all replicate plates at all dilutions with usable counts

d. Divide the sum of PFU by the sum of the corresponding undiluted sample volumes to obtain PFU/mL

Figure X. Example of countable plaques on a *Bacteriophage MS2* plate

v. Sample Data and Equations

To back calculate the concentration of the enumerated *Bacteriophage MS2*

Example DAL data

Dilution	PFU / plate (for each duplicate plate)	Volume of *undiluted* spiking suspension (mL)
Undiluted	TNTC, TNTC	0.5 mL
1 : 10	35, 37	0.05
1 : 100	0, 3	0.005
1 : 1,000	0, 0	0.0005

1. Undiluted spiking suspension PFU / mL = $(PFU_1 + PFU_2 \ldots PFU_n)/(V_1 + V_2 \ldots V_n)$
   a. Where:

PFU = number of plaque forming units from plates of all countable sample dilutions (dilutions with 1 or more PFU per plate, excluding dilutions with all TNTC or all zeros (0)

V = volume of undiluted sample in all plates with countable plaques
n = number of useable counts

For given data: (35 + 37 + 0 + 3)/(0.05 + 0.05 + 0.005 + 0.005) = 75/0.11 = 682 PFU / mL

In this example, the undiluted spiking suspension contains approximately 682 PFU per mL, the 1:10 dilution contains approximately 68.2 PFU per mL, the 1 :100 dilution contains approximately 6.82 PFU per mL, and the 1 : 1000 dilution contains approximately 0.682 PFU per mL

2. Bacteriophage Reactor Procedure

Materials

TSB with Antibiotics
TSB without Antibiotics
60 mL Syringes
50 mL Centrifuge Tube
Test tubes
E. coli Stock (ATCC 700891)
TSB bottom and Top Agar Plates
Microcentrifuge Tubes
Autoclaved Deionized Water
Autoclaved 100 mL beakers

a. Sample Preparation
    i. Create a MS2 Bacteriophage solution to run through the microreactor
        1. Make 60 mL of Autoclaved DI water and inoculate with desired volume of *Bacteriophage MS2* stock to a sterile beaker
        2. Spin on a spin plate for solution to be well mixed
            a. Take 100 uL for positive control sample
        3. Uptake remaining liquid into the sterile 60 mL syringe and load into microreactor for testing

b. Sample Collection
    i. Upon washout time (3 residence times) collect at least 100 uL of sample into 50 mL centrifuge tube

c. Enumeration of Samples
    i. Vortex collected sample
    ii. Serially dilute sample and plate all dilutions D0-DX in duplicates

d. Back calculation
    i. Quantify positive control sample and convert to log subtract log calculation from the plate from positive control sample concentration

Cryptosporidium parvum Enumeration by Vital Dye Assay Method

1. **Purpose**
   This protocol describes the process to determine the concentration of oocysts/mL from an effluent sample.

2. **Materials**
   Cryptosporidium stock
   Hank's Balanced Salt Solution
   Acidified Hank's Balanced Salt Solution
   Propidium Iodide (PI)
   DAPI
   Autoclaved DI Water
   Bovine Bile Solution
   Sodium Hydrogen Carbonate Solution
   Microcentrifuge Tubes
   Neubauer Hemocytometer

3. **Reagent Preparation**
   a. Hank's Balanced Salt Solution
      i. 1 L deionized water
      ii. 48 mg Na_2HPO_4
      iii. 400 mg KCL
      iv. 350 mg Na_2CO_3
      v. 8 g NaCl

      Add materials to 1 L media bottle, shake to incorporate, adjust pH to 7.4, open a quarter-turn and autoclave for 15 minutes at 121°C.

   b. DAPI preparation
      i. DAPI powder
      ii. Absolute methanol

      In the dark, Dilute DAPI powder to desired concentration, literature reference (2mg/1mL of Absolute methanol). Aliquot into several microcentrifuge tubes to prevent contamination.

   c. PI Preparation
      i. PI powder
      ii. Autoclaved DI water

      In the dark dilute the PI powder to desired concentration, literature reference (1mg/1mL DI). Aliquot into several microcentrifuge tubes to prevent contamination.

   d. Bovine Bile Solution
      i. 0.1 g bovine bile
      ii. 10 mL HBSS

      Combine materials into a 50 mL centrifuge tube and vortex to combine.

e. Sodium Hydrogen Carbonate Solution
    i. 0.44 g Na_2CO_3
    ii. 100 mL DI water

Combine materials into a 250 L media bottle and shake to mix.

4. Procedures

a. Preparation of *Cryptosporidium* Sample
    i. Create a Crypto solution to run through the microreactor
        1. Make 60 mL of Autoclaved DI water and inoculate with desired volume of Crypto stock to a sterile beaker
        2. Spin on a spin plate for solution to be well mixed
            a. Take 100 uL for positive control sample
        3. Uptake remaining liquid into the sterile 60 mL syringe and load into microreactor for testing

b. Preincubation with Acidified HBSS

To disintegrate the cell wall of Crypto to allow PI to permeate cell

    i. Sterilize the countertop by wiping down with ethanol (70%)
    ii. Take 100uL of oocyst stock suspension into microcentrifuge tube
    iii. Add by 1 mL of Acidified HBSS (20 mL HBSS + 200 uL 1M HCL, pH: 2.74) to sample
    iv. Incubate at 37° for 1 hour
    v. After incubation, wash cells in microcentrifuge at 4000 rpm for 3 minutes
    vi. Aspirate the supernatant
        1. The pellet will be invisible, be careful not to uptake all the liquid
    vii. Resuspend the sample with 1 mL of HBSS
    viii. Repeat this procedure for a total of 3 times

c. Vital Dye Staining
    i. Take 100 uL of sample and add 10 uL of DAPI and 10 uL of PI to microcentrifuge tube
    ii. Incubate sample for 2 hours and wash thrice with 100 uL of HBSS, resuspend with HBSS when done

d. In vitro Excystation
    i. Take Sample from Vital Dye Staining Incubation
    ii. Add 200 uL of Bovine Bile Solution (made <30 minute prior to excystation)
    iii. Add 50 ul Sodium Hydrogen Carbonate
    iv. Vortex and incubate for 3-4 hours
    v. Wash sample thrice with HBSS

e. Fluorescence Microscopy
    i. Take 6 uL of sample each well on the hemocytometer
    ii. Add carefully to hemocytometer, ensure there are no air bubbles
    iii. View in microscope

f. Hemocytometer Counting

i. At the inlet of the hemocytometer add 6 uL of the sample slowly to ensure no air bubbles
ii. Let the hemocytometer stand for ~ 3 minutes to settle prior to viewing
iii. Place hemocytometer on the microscope stage
iv. Adjust the objective so that one of the large squares is showing
v. Count all cells found in the corner cells ONLY – use a systematic fashion when counting e.g. side-to-side, up-and-down
vi. Be sure adhere to border rule as well (See Hemocytometer Protocol)

g. Calculations and Equations

i. To calculate the concentration of cells from your hemocytometer counts:

ii. Average the number of cells counted in the corner squares:

$$\text{Average cells counted (n)} = \frac{S_1\ \#\ of\ cells + S_2\ \#\ of\ cells + S_3\ \#\ of\ cells + S_4\ \#\ of\ cells}{\#\ of\ counted\ squares}$$

iii. Now solve for the # of cells per large square:
We know that the volume of the large square is 0.1 mm^3

$$\frac{\#\ of\ cells}{Large\ Square} = \frac{n\ (average\ cells\ counted)}{0.1\ mm^3\ (volume\ of\ the\ large\ square)}$$

iv. To convert that to # of cells/ 1 mL:

$$\frac{\#\ of\ cells}{1\ mL} = \frac{n\ (average\ cells\ counted)}{0.1\ mm^3\ (volume\ of\ the\ large\ square)}\ \text{x}\ \frac{1000\ mm^3}{1\ cm^3}\ \text{x}\ \frac{1\ cm^3}{1\ mL}$$

5. *Cryptosporidium* Reactor Procedure

Materials

Cryptosporidium stock
Hank's Balanced Salt Solution
Acidified Hank's Balanced Salt Solution
Propidium Iodide (PI)
DAPI
Autoclaved DI Water
Bovine Bile Solution
Sodium Hydrogen Carbonate Solution
Microcentrifuge Tubes
Neubauer Hemocytometer

a. Sample Preparation
   Preparation of *Cryptosporidium* Sample
   i. Create a Crypto solution to run through the microreactor
      1. Make 60 mL of Autoclaved DI water and inoculate with desired volume of Crypto stock to a sterile beaker
      2. Spin on a spin plate for solution to be well mixed
         a. Take 100 uL for positive control sample
      3. Uptake remaining liquid into the sterile 60 mL syringe and load into microreactor for testing

b. Sample Collection
   i. Upon washout time (3 residence times) collect at least 200 uL of sample into 50 mL centrifuge tube

c. Enumeration of Samples
   i. Vortex collected sample
   ii. Follow Vital Dye Assay Protocol
   iii. Follow Excystation Protocol
   iv. Fluorescence Microscopy

Appendix B

B1. Microreactor Flow Rates

Volumetric flow rates were determined by the following equation:

$$Q = \frac{V}{t}$$

Where…

Q = Volumetric Flow Rate
V = Reactor Internal Volume t = Residence Time

Table B.1. Volumetric Flow Rates for Given Residence Times in UV Microreactor

Residence Time (sec)	Volumetric Flow Rate (mL/min)	Microbe Tested
1.57	65.00	*E. coli* *C. parvum* *Bacteriophage MS2*
5	20.55	*E. coli*
8	12.83	*E. coli*
15	6.86	*E. coli* *C. parvum* *Bacteriophage MS2*
30	3.43	*E. coli* *C. parvum* *Bacteriophage MS2*
120	0.858	*C. parvum* *Bacteriophage MS2*
360	0.286	*C. parvum* *Bacteriophage MS2*
480	0.214	*Bacteriophage MS2*