USING NOVEL TOOLS FOR RISK REDUCTION IN PRE- AND POST-HARVEST FOOD SAFETY

by

Alyssa Kelly

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the Master of Science in Food Science

Spring 2022

USING NOVEL TOOLS FOR RISK REDUCTION

IN PRE- AND POST-HARVEST FOOD SAFETY

by

Alyssa Kelly

Approved: _____

Kalmia E. Kniel, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____

Erin Connor, Ph.D.
Chair of the Department of Animal and Food Sciences

Approved: _____

Calvin Keeler, Ph.D.
Dean of the College of Agriculture & Natural Resources

Approved: _____

Louis F. Rossi, Ph.D.
Vice Provost for Graduate and Professional Education and
Dean of the Graduate College

ACKNOWLEDGMENTS

The genetic sequencing projects were funded by the Center of Produce Safety (2019) and in part supported by a USDA-funded Center of Excellence CONSERVE project (www.conservewaterforfood.org). CONSERVE was awarded to the University of Maryland School of Public Health and is supported by the United States Department of Agriculture-National Institute of Food and Agriculture, Grant number 20166800725064. The ZVI filtration project was also funded by the Center for Produce Safety. The chlorine dioxide project was funded by the CAP grant. Thank you to all of the organizations for allowing me to complete these research projects.

First, I want to thank Dr. Kniel for the support, guidance and patience throughout this journey towards my master's degree. I have expanded my knowledge of food safety and microbiology working in your lab for over four years. I would also like to thank my committee Dr. Rolf Joerger, Dr. Haiqiang Chen, and Dr. Manan Sharma for all your wisdom and continuing support. Thank you to my lab mates Shani Craighead, Brienna Anderson, Alexis Omar, Pushpinder Kaur Litt and Adrienne Shearer.

Finally, I would like to thank my family for all of their support, time and understanding through this time in my life. I would not have been able to do this without all of these people, I am truly grateful to be able to surround myself with such amazing people.

I am continuing my love for food microbiology at Romer Labs. I have been lucky enough to be able to work for the over the past few months as their lead

microbiologist and production scientist. I develop lateral flow detection systems for pathogens such as *Listeria, Salmonella, E. coli and Campylobacter.* I am excited to take what I have learned throughout my time at the University of Delaware into my future career.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Cyclospora cayetanensis is a lesser studied member of the Apicomplexan family of protozoa, which includes more well studied protozoan genera like *Cryptosporidium*, *Eimeria*, and *Toxoplasma*. However, *C. cayetanensis* is an important protozoan parasite that continues to cause outbreaks of gastroenteritis associated with contaminated fresh produce. *Cyclospora* oocysts may be transmitted through contaminated surface water, thereby making reliable detection imperative for public health and produce safety. *Cyclospora* is still considered an emerging pathogen by many, due to the limited understanding and large data gaps surrounding the transmission and survival of oocysts in the environment limitations in detection of oocysts. Polymerase chain reaction (PCR) analysis is used for detection of a presumptive positive water sample. Filtration using the ZVI-sand filtration achieved a 4.30-log reduction of *Cryptosporidium parvum* compared to 1.82 log reduction using sand filtration. When comparing the two filtration systems, there was a significant difference in efficacy ($p < 0.05$) with ZVI-sand filtration yielding larger reductions of *C. parvum* oocysts. For reduction of *Eimeria tenella* oocysts, ZVI-sand filters achieved a 6-log reduction, whereas sand-only filtration achieved a 2.3-log reduction. There was a significant difference ($p < 0.05$) in ability to inactivate and remove *E.* tenella oocysts, the ZVI-sand filtration performed better compared to the

sand alone filtration. This study investigated the effects of gaseous chlorine dioxide at varying treatment times on *Cryptosporidium parvum* on produce, baby cut carrots and grape tomatoes. Grape tomatoes (approx.40g) and baby-cut carrots (approx. 35g) were spot inoculated (10^6 oocysts) with 100-µl of *C. parvum* suspension in 10-µl droplets randomly distributed on the produce samples. Inoculated produce samples were treated with 50 g of each gaseous chlorine dioxide precursor for 0, 1 and 3 hours in a 35-L enclosed chamber. Significant reductions were shown at both 1 hour and 3-hour treatment times for both produce types. The results of this work will facilitate the development of novel on-farm filtration technology and guidelines for commercial applications to control parasitic pathogens in agricultural water, thus improving the safety of produce and control of protozoa in agricultural water. A total of seventy-two water samples were collected regularly from various sites in Delaware and Maryland over the course of 17 months (June 2017-October 2018). DNA Primer Sequences for *Cryptosporidium* Genus-specific PCR Amplification (18S rRNA) used, followed by further testing using *Vsp*I and *Dra*II restriction fragment length polymorphism enzyme for speciation confirmation. The nested PCR amplicons were digested with one unit of *Vsp*I for distinction between *C. parvum* and *C. hominis*. For distinction of *C. parvum, C. baileyi* and *C. serpenti*, the nested PCR amplicon were digested with one unit *Dra*II. Twenty-four out of seventy-two water samples were confirmed to contain *Cryptosporidium parvum*. Through further testing using restriction fragment length polymorphism analysis, one water sample was confirmed to harbor *C. cayetanensis* or *E. neischulzi,* which have genetic similarities to *Cryptosporidium* species. A single

sample was identified to be either *C. baileyi* or *C. serpenti,* which do not infect humans. Knowledge of parasitic organisms in potential irrigation water sources are essential for correct implementation of water testing and water treatment.

Chapter 1

WHAT IS *CYCLOSPORA*? WHAT WE KNOW AND WHAT WE DO NOT KNOW

1.1 What is Cyclospora?

Cyclosporiasis is caused by consuming food or drink containing *Cyclospora cayetanensis*. Symptoms of cyclosporiasis in humans include watery diarrhea, stomach pain, bloating, vomiting and other flu-like symptoms. *C. cayetanensis* is transmitted through the fecal oral route and typically colonizes in the small intestine. The incubation period of *C. cayetanensis* infection ranges from 2 to 11 days with the median incubation period of 7 days (Almeria *et al.,* 2019).

Cyclospora species are protozoan in the phylum Apicomplexa, class Sporozoasida, subclass Coccidiasina, order Eimeriorina, and family Eimeriidae. *C. cayetanensis* was first described and named in 1994 (Ortega *et al.,* 1994). There are currently twenty-two species of *Cyclospora* that have been isolated from various animals including rodents, insects, snakes, moles and humans. *C. cayetanensis* is the only *Cyclospora* species known to infect humans (Ortega & Sanchez, 2010). The overall prevalence of *C. cayetanensis* in humans worldwide is 3.55%, with the highest prevalence in immunocompromised individuals (Li *et al.,* 2020).

Infection with *C. cayetanensis* are seasonal, with spikes in rainy seasons and the summer. Produce most associated with *C. cayetanensis* infections are berries, basil and cilantro (Hadjilouka and Tsaltas, 2020). *Cyclospora* can also be transmitted by consumption of contaminated water or produce irrigated or washed with contaminated water. Cyclosporiasis is often associated with underdeveloped or developing countries due to high population density, lower economic conditions and poor sanitation (Fludkowska *et al.,* 2017). Prior to 2018, individuals from industrialized nations, typically developed cyclospoiasis during international travel to developing countries or from contaminated water or produce (Hadjilouka and Tsaltas*,* 2020).

The detection and investigation of foodborne illness outbreaks of cyclosporiasis have been stifled due to the lack of genetic information available. The entire genome of *C. cayetanensis* has been sequenced which concluded that *C. cayetanensis* is genetically very similar to *Eimeria tenella* in both genomic features and metabolic capabilities. Through whole genome sequencing, the major differences between *C. cayetanensis* and other apicomplexans are based on amino acid metabolism and post-translational modifications of proteins (Liu *et al.,* 2016).

1.2 Domestically Acquired *Cyclospora* Outbreaks

Cyclospora cayetanensis has been found to be linked to outbreaks with produce samples such as raspberries, basil, cilantro, snow peas and mesclun lettuce (Hadjilouka and Tsaltas, 2020). Outbreaks of cyclosporiasis commonly increases in

the spring and summer months for domestically acquired outbreaks. Between May – August 2021, there were 1,020 cases of cyclosporiasis in the United States including 70 hospitalizations. There were two large multistate *Cyclospora* outbreaks in 2021. An outbreak in August 2021 which included 40 cases and one in July 2021 which included 130 cases. The food sources of the outbreaks were unable to be determined. Epidemiological surveys linked a variety of leafy green products, however, all samples that were tested were negative for *Cyclospora* therefore a specific product could not be identified as the source (CDC, 2021).

In 2020, there were 1,241 reported cases of cyclosporiasis including 90 hospitalizations. A large outbreak accounted for 701 of those cases. Epidemiologic evidence and product traceback to indicated the source of the outbreak was bagged salad mix that contained iceberg lettuce, carrots, and red cabbage. There was a product recall on June 27, 2020 from Fresh Express due to contaminated salad products produced at the Streamwood, IL facility (CDC, 2020).

In 2019, 2,408 laboratory confirmed cases of cyclosporiasis were reported to the CDC and 144 people were hospitalized. The largest *Cyclospora* outbreak in 2019 was linked to fresh basil from Siga Logistics de RL de CV of Morelos, Mexico. There were 241 laboratory confirmed cases across eleven states and included six hospitalizations. A recall of potentially affected basil occurred on July 24, 2019 (CDC, 2019).

A total of 2,299 laboratory confirmed cases of cyclosporiasis occurred in 2018 including 160 hospitalizations. One-third of the illnesses in 2018 were part of two

multistate outbreaks. The first outbreak was epidemiologically linked to pre-packaged Del Monte Fresh Produce vegetable trays, containing broccoli, cauliflower, carrots and dill dip, sold at convenience store chains. This outbreak included 250 laboratory-confirmed cases with eight hospitalizations from four states. Traceback to a specific food product within the pre-packaged vegetable tray was not possible (CDC, 2018a). In May through July 2018, a multistate outbreak occurred resulting in 511 laboratory-confirmed cases of *Cyclospora* infections from consumption of salads from McDonald's restaurants in the Midwest. Epidemiological traceback indicated that salads were the likely source of this outbreak and on July 13, 2018 McDonald's voluntarily stopped selling salads. The FDA was able to sample an unused Fresh Express (Steamwood, IL) package of romaine lettuce and carrot mix confirming the presence of *Cyclospora* (CDC, 2018b).

Many cases of cyclosporiasis could not be directly linked to an outbreak due to the lack of validated molecular typing tools for *C. cayetanensis*. A significant increase in the number of reported cyclosporiasis occurred after 2017 due to the creation and use of a multiplex molecular test, the BioFire FilmArray GI panel.

1.3 *Cyclospora* Survival in the Environment

Cyclospora oocysts mature in the environment for 7 to 10 days before sporulating, after that time period oocysts are excyst and become infective (Ortega *et al.,* 1994). There is a lack of knowledge about how *C. cayetanensis* is transported in the environment and which factors inhibit or promote sporulation. In a comprehensive

molecular survey of *C. cayetanensis*, data suggested that irrigation water, soil and vegetables are possible sources of infection for humans (Giangaspero *et al.,* 2015).

1.4 Detection Methodologies

There is a lack of technology for detection of *Cyclospora* in clinical samples as well as in environmental samples. Currently there are no serological tests to identify human exposure to *Cyclospora,* however specific IgG and IgM antibodies in individuals with oocysts were tested by enzyme-linked immunosorbent assay (ELISA) (Almeria *et al.,* 2019). Microscopy including use of a modified acid-fast stain is used to screen for *Cyclospora, Cryptosporidium,* and *Cystoisospora*. *Cyclospora* can also be identified microscopically using autofluorescence; when exposed exposure to 365nm UV light *Cyclospora* appears blue and when exposed to 450-490nm UV light *Cyclospora* green. Whereas *Cryptosporidium* appears violet when exposed to 365nm UV light and green under 405-436nm UV light (Ortega *et al.,* 1998; Varea *et al.,* 1998).

Research in the area of molecular detection methods to identify and isolate *C. cayetanensis* has gained momentum in recent years. Several conventional, nested, real-time and quantitative PCR assays have been developed. Nested PCR detection utilizes specific primers for small subunit rRNA or ITS regions able to detect one to ten oocysts (Li *et al.,* 2007). Quantitative PCR can be used for *C. cayetanensis* detection with specific primers and probe or using the 18S ribosomal gene sequence (Varma *et al.,* 2003). Muliplex real-time PCR used in conjunction with a T4 phage internal

control has been recently discovered as a method for molecular diagnostic distinction between *Cryptosporidium parvum, Giardia lamblia,* and *C. cayetanensis* in human stool (Shin *et al.,* 2018) There is currently only one commercial molecular diagnostic test available for clinical sample testing, the BioFire FilmArray GI panel. This detection system was determined to have 100% sensitivity for 12/22 targets, including *Cyclospora cayetanensis* and *Cryptosporidium* spp., and ≥94.5% sensitivity for an additional 7/22 targets (Buss *et al.,* 2015. The sensitivity and specificity of the FilmArray GI Panel, have resulted in faster identification of illness, quicker medical intervention and may lead to the ability to promptly reduce transmission during an outbreak.

The research fields of whole genome sequencing of *Cyclospora cayetanensis* as well as mitochondrial and apicoplast genome sequencing continue to advance. Exploration of apicoplast and mitochondrial genomes resulted in the discovery that *C. cayetanensis* is highly similar to *Eimeria* spp., specifically *E. tenella* (Tang *et al.,* 2015). Whole genome sequence analysis is primarily used in epidemiological traceback to characterize and compare microbial genomes. Due to genome-comparing resources available with the whole genome sequencing, genetic heterogeneity confounds allow for the utility of traditional sequence-typing and phylogenetic approached for aiding in epidemiological trace-back (Barratt *et al.,* 2019). Whole genome sequencing can link outbreaks that come from the same source and can shed new light on the virulence, antimicrobial resistance and foodborne microbial ecology.

Detection of protozoan oocysts from food samples, in particular produce commodities, occurs by carefully washing the samples coupled with a molecular technique such as qPCR and/or multiplex qPCR. While fewer studies have focused specifically on *C. cayetanensis*, the parasite has been successfully detected in water and wastewater using current methods to recover *Cryptosporidium* oocysts (Quintero-Betancount *et al.,* 2002). *Cryptosporidium* oocysts are routinely detected by filtration or purification via immunomagnetic separation and subsequent labeling of oocysts with specific fluorochrome and differential interference microscopy detection (Giangaspero and Gasser, 2019).

1.5 Technologies for Reduction of *C. cayetanensis*

Potential technologies for the reduction of *C. cayetanensis* and surrogate organisms, *Cryptosporidium* and *Eimeria*, have been studied recently. High hydrostatic pressure, UV light, microwave heating, nanoparticles and more technologies have been evaluated for parasite reduction potential for protection of public health from *C. cayetanensis*.

High hydrostatic pressure and UV light treatment were evaluated for their ability to reduce *Eimeria acervulina*, a surrogate for *C. cayetanensis*. In this study, raspberries and basil were inoculated with sporulated *E. acervulina* at high (10^6 oocysts) and low (10^4 oocysts) levels and treated with UV (up to 261mW/cm^2) or HPP (550MPa at 40°C for 2 minutes). Broiler chickens that were fed oocysts from low level UV-treated raspberries were asymptomatic but shed oocysts. Whereas, when

birds were fed low levels of oocysts from contaminated, UV-treated basil the birds were asymptomatic and did not shed oocysts. Broiler chickens fed HPP-treated raspberries and basil were asymptomatic and did not shed oocysts. Results of this study suggested that both UV radiation and HPP may be able to reduce the risk of cyclosporiasis infection, however; HPP was found to be more effective compared to UV radiation (Kniel *et al.,* 2007).

Microwave heating was evaluated for the reduction in viability of *Cryptosporidium parvum* oocysts and on the sporulation of *Cyclospora cayetanensis* oocysts at various cooking times at 100% power. *C. parvum* was completely inactivated in less than 20 seconds of microwave heating, whereas *C. cayetanensis* was observed up to 45 seconds. Using conventional heating sporulation of *Cyclospora* was found to be inhibited when oocysts were exposed to 70° for 15 minutes (Sathyanarayanan and Ortega, 2006). *Cryptosporidium* oocysts were non-infectious when heated to 72.4°C for 1 minute (Harp *et al.,* 1996).

A 2018 study observed the effect of magnesium oxide nanoparticles (NPs) on the reduction of *C. cayetanensis* oocysts. Magnesium oxide nanoparticles are a potentially useful tool in food as a bactericide. This study examined dose a range of 1.25-25mg/mL of MgO NPs exposure for pre- and post-sporulated *C. cayetanensis* oocysts. Sporulation failure occurred for the treated oocysts at a level of ≥90% after 24 hours and 72 hours of incubation with 15 and 12.5mg/mL, respectively. The conclusion of this study determined that MgO NPs had a significant anti-*Cyclospora*

effect on both unsporulated and sporulated oocysts and could be a preventative agent in food and water disinfectant treatment (Hussein *et al.,* 2018).

Cryptosporidium and *Eimeria* are common surrogates used in the evaluation for technologies to evaluate potential reduction of *Cyclospora* due to the lack of detection methods. *Eimeria* is frequently used as a surrogate for *Cyclospora cayetanensis* because it offers a biologically and genetically similar organism to experiment with novel technologies. Table 1 shows potential treatments for the reduction of *Cryptosporidium* oocysts on food matrices in variable matrices.

Table 1: List of potential treatments for reduction of *Cryptosporidium* oocysts on food matrices and different water types.

	Reduction:	*Treatment Time:*	*Concentration:*	*Matrix:*	*Source:*
Gaseous Chlorine Dioxide	2.60 log 3.31 log	20 Min	4.1 mg/L	Basil Lettuce	Ortega et al., 2008
Cold Atmospheric Plasma	0.84 log 1.23 log 2.03 log	30 Sec 90 Sec 180 Sec	1.42 × 10-3 m3/s	Cilantro	Craighead et al., 2020
Ozone	49% inactivated 92% inactivated	0 min 10 min	2.5 mg/L	Drinking Water	Wohlsen et al., 2007
Free Chlorine	3.7 log	10 hours	20mg/ml	Swimming Pool Water	Shields et al., 2009
Ceramic Filters Impregnated with Silver Nanoparticles	1.5-2.1 log	Flow rate: 1.5L/h	N/A	Drinking Water	Abebe et al., 2015
Pulsed UV Light	2-3 log	300 µs	4 J/cm2	Raspberries	Le Goff et al., 2015
Microfiltration	100%	Transmembrane pressure: 155kPa	Pour sizes:0.8 and 1.4 mm	Apple Juice	Zhao et al., 2014

1.6 Conclusion

An increase in the annual cases of cyclosporiasis in the United States has resulted in an increase in research into detection methods and technologies for the reduction of *C. cayetanensis.* There is a lack of knowledge regarding detection methods pertaining to water or soil, prevalence and factors associated with *C.*

cayetanensis contamination of soil, and methods for controlling *Cyclospora* in the environment (Totton *et al.,* 2021). Continued research on cyclosporiasis in humans, food products and water sources would lead to a better understanding of the organism's characteristics, reduction, and prevention. Further research to establish *in vitro* and *in vivo* methods for cultivation *C. cayetanensis* is needed along with detection methods and genetic traceback methods should be further developed to help monitor cyclosporiasis infection to better protect public health.

REFERENCES

Abebe, Lydia S et al. "Point-of-Use Removal of Cryptosporidium parvum from Water: Independent Effects of Disinfection by Silver Nanoparticles and Silver Ions and by Physical Filtration in Ceramic Porous Media." *Environmental science & technology* vol. 49,21 (2015): 12958-67. doi:10.1021/acs.est.5b02183

Almeria, Sonia et al. "*Cyclospora cayetanensis* and Cyclosporiasis: An Update." *Microorganisms* vol. 7,9 317. 4 Sep. 2019, doi:10.3390/microorganisms7090317

Barratt, Joel L N et al. "Genotyping genetically heterogeneous *Cyclospora cayetanensis* infections to complement epidemiological case linkage." *Parasitology* vol. 146,10 (2019): 1275-1283. doi:10.1017/S0031182019000581

Buss, Sarah N et al. "Multicenter evaluation of the BioFire FilmArray gastrointestinal panel for etiologic diagnosis of infectious gastroenteritis." *Journal of clinical microbiology* vol. 53,3 (2015): 915-25. doi:10.1128/JCM.02674-14

Center for Disease Control (2021). "Cyclosporiasis Outbreak Investigations - United States, 2021." *Centers for Disease Control and Prevention*, Centers for Disease Control and Prevention, 30 Sept. 2021, https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/2021/seasonal/index.html.

Center for Disease Control (2020). "Cyclosporiasis Outbreak Investigations - United States, 2020." *Centers for Disease Control and Prevention*, Centers for Disease Control and Prevention, 24 Sept. 2020, https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/2020/.

Center for Disease Control (2019). "Cyclosporiasis Outbreak Investigations - United States, 2019." *Centers for Disease Control and Prevention*, Centers for Disease Control and Prevention, 2 Dec. 2019, https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/2019/weekly/index.html.

Center for Disease Control (2018a). "Cyclosporiasis Outbreak Investigations - United States, 2018." *Centers for Disease Control and Prevention*, Centers for Disease Control and Prevention, 17 Sept. 2018, https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/2018/a-062018/index.html.

Center for Disease Control (2018b). “Cyclosporiasis Outbreak Investigations - United States, 2018.” *Centers for Disease Control and Prevention*, Centers for Disease Control and Prevention, 17 Sept. 2018, https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/2018/b-071318/index.html.

Craighead, Shani et al. “Cold Atmospheric Plasma Jet Inactivates Cryptosporidium parvum Oocysts on Cilantro.” *Journal of food protection* vol. 83,5 (2020): 794-800. doi:10.4315/0362-028X.JFP-19-442

Giangaspero, Annunziata, and Robin B Gasser. “Human cyclosporiasis.” *The Lancet. Infectious diseases* vol. 19,7 (2019): e226-e236. doi:10.1016/S1473-3099(18)30789-8

Giangaspero, Annunziata et al. “Molecular detection of Cyclospora in water, soil, vegetables and humans in southern Italy signals a need for improved monitoring by health authorities.” *International journal of food microbiology* vol. 211 (2015): 95-100. doi:10.1016/j.ijfoodmicro.2015.07.002

Hadjilouka, Agni, and Dimitris Tsaltas. “Cyclospora Cayetanensis-Major Outbreaks from Ready to Eat Fresh Fruits and Vegetables.” *Foods (Basel, Switzerland)* vol. 9,11 1703. 20 Nov. 2020, doi:10.3390/foods9111703

Harp, J A et al. “Effect of pasteurization on infectivity of Cryptosporidium parvum oocysts in water and milk.” *Applied and environmental microbiology* vol. 62,8 (1996): 2866-8. doi:10.1128/aem.62.8.2866-2868.1996

Hussein, Eman M et al. “Antiprotozoal activity of magnesium oxide (MgO) nanoparticles against Cyclospora cayetanensis oocysts.” *Parasitology international* vol. 67,6 (2018): 666-674. doi:10.1016/j.parint.2018.06.009

Kniel, Kalmia E et al. “High hydrostatic pressure and UV light treatment of produce contaminated with Eimeria acervulina as a Cyclospora cayetanensis surrogate.” *Journal of food protection* vol. 70,12 (2007): 2837-42. doi:10.4315/0362-028x-70.12.2837

Kłudkowska, M., Pielok, Ł., Frąckowiak, K. *et al.* Intestinal coccidian parasites as an underestimated cause of travellers’ diarrhoea in Polish immunocompetent patients. *Acta Parasit.* **62,** 630–638 (2017). https://doi.org/10.1515/ap-2017-0077

Le Goff, L et al. “Pilot-Scale Pulsed UV Light Irradiation of Experimentally Infected Raspberries Suppresses Cryptosporidium parvum Infectivity in Immunocompetent Suckling Mice.” *Journal of food protection* vol. 78,12 (2015): 2247-52. doi:10.4315/0362-028X.JFP-15-062

Li, Junqiang et al. “Advances in Cyclosporiasis Diagnosis and Therapeutic Intervention.” *Frontiers in cellular and infection microbiology* vol. 10 43. 11 Feb. 2020, doi:10.3389/fcimb.2020.00043

Li, Junqiang et al. “*Cyclospora cayetanensis* infection in humans: biological characteristics, clinical features, epidemiology, detection method and treatment.” *Parasitology* vol. 147,2 (2020): 160-170. doi:10.1017/S0031182019001471

Liu, S., Wang, L., Zheng, H. *et al.* Comparative genomics reveals *Cyclospora cayetanensis* possesses coccidia-like metabolism and invasion components but unique surface antigens. *BMC Genomics* **17,** 316 (2016). https://doi.org/10.1186/s12864-016-2632-3

Ortega, Ynés R, and Roxana Sanchez. “Update on Cyclospora cayetanensis, a food-borne and waterborne parasite.” *Clinical microbiology reviews* vol. 23,1 (2010): 218-34. doi:10.1128/CMR.00026-09

Ortega, Ynes R et al. “Efficacy of gaseous chlorine dioxide as a sanitizer against Cryptosporidium parvum, Cyclospora cayetanensis, and Encephalitozoon intestinalis on produce.” *Journal of food protection* vol. 71,12 (2008): 2410-4. doi:10.4315/0362-028x-71.12.2410

Ortega, Y R et al. “Cyclospora cayetanensis.” *Advances in parasitology* vol. 40 (1998): 399-418. doi:10.1016/s0065-308x(08)60128-1

Ortega, Y R et al. “A new coccidian parasite (Apicomplexa: Eimeriidae) from humans.” *The Journal of parasitology* vol. 80,4 (1994): 625-9.

Quintero-Betancourt, Walter et al. “Cryptosporidium parvum and Cyclospora cayetanensis: a review of laboratory methods for detection of these waterborne parasites.” *Journal of microbiological methods* vol. 49,3 (2002): 209-24. doi:10.1016/s0167-7012(02)00007-6

Sathyanarayanan, Lakshmi, and Ynes Ortega. “Effects of temperature and different food matrices on Cyclospora cayetanensis oocyst sporulation.” *The Journal of parasitology* vol. 92,2 (2006): 218-22. doi:10.1645/GE-630R.1

Shields, Joan M et al. “The effect of cyanuric acid on the disinfection rate of Cryptosporidium parvum in 20-ppm free chlorine.” *Journal of water and health* vol. 7,1 (2009): 109-14. doi:10.2166/wh.2009.008

Shin, Ji-Hun et al. “Development of Molecular Diagnosis Using Multiplex Real-Time PCR and T4 Phage Internal Control to Simultaneously Detect Cryptosporidium parvum, Giardia lamblia, and Cyclospora cayetanensis from Human Stool Samples.” *The Korean journal of parasitology* vol. 56,5 (2018): 419-427. doi:10.3347/kjp.2018.56.5.419

Tang, Kevin et al. “Genetic similarities between Cyclospora cayetanensis and cecum-infecting avian Eimeria spp. in apicoplast and mitochondrial genomes.” *Parasites & vectors* vol. 8 358. 8 Jul. 2015, doi:10.1186/s13071-015-0966-3

Totton, S C et al. “A scoping review of the detection, epidemiology and control of *Cyclospora cayetanensis* with an emphasis on produce, water and soil.” *Epidemiology and infection* vol. 149 e49. 28 Jan. 2021, doi:10.1017/S0950268821000200

Varea, M et al. “Fuchsin fluorescence and autofluorescence in Cryptosporidium, Isospora and Cyclospora oocysts.” *International journal for parasitology* vol. 28,12 (1998): 1881-3. doi:10.1016/s0020-7519(98)00146-5

Varma, Manju et al. “Detection of Cyclospora cayetanensis using a quantitative real-time PCR assay.” *Journal of microbiological methods* vol. 53,1 (2003): 27-36. doi:10.1016/s0167-7012(02)00209-9

Wohlsen, T et al. “The efficiency of ozonated water from a water treatment plant to inactivate Cryptosporidium oocysts during two seasonal temperatures.” *Journal of water and health* vol. 5,3 (2007): 433-40. doi:10.2166/wh.2007.039

Zhao, Dongjun et al. “Efficient reduction of pathogenic and spoilage microorganisms from apple cider by combining microfiltration with UV treatment.” *Journal of food protection* vol. 78,4 (2015): 716-22. doi:10.4315/0362-028X.JFP-14-452

Chapter 2

CONFIRMATION OF THE PRESENCE OF CYCLOSPORA CAYETANENSIS IN PRESUMPTIVE POSITIVE ENVRIRONMENTAL WATER SAMPLES

2.1 Abstract

Cyclospora cayetanensis is a lesser studied member of the Apicomplexan family of protozoa, which includes more well studied protozoan genera like *Cryptosporidium*, *Eimeria*, and *Toxoplasma*. However, *C. cayetanensis* is an important protozoan parasite that continues to cause outbreaks of gastroenteritis associated with contaminated fresh produce. *Cyclospora* oocysts may be transmitted through contaminated surface water, thereby making reliable detection imperative for public health and produce safety. Polymerase chain reaction (PCR) analysis is used for detection of a presumptive positive water sample. Further analysis of that PCR amplicon by sequencing provides evidence of the close familial genetic relationship of these protozoa, as numerous samples positive by PCR were not identified as *Cyclospora* following phylogenetic analysis of amplicon sequencing. This analysis is complex.

2.2 Introduction

Cyclospora cayetanensis is an intracellular protozoan parasite infectious to humans resulting in a gastrointestinal illness. *Cyclospora* is recognized as an emerging foodborne and waterborne pathogen. *Cyclospora* infects the small intestine and

typically causes symptoms including watery diarrhea, loss of appetite, weight loss, stomach pain, bloating, increased gas, nausea and fatigue. The infectious dose of *C cayetanensis* is unknown but considered to be very low (Hadjilouka & Tsaltas, 2020). Gaps in the scientific understanding of this protozoan parasite are great, including in areas of detection, environmental persistence and transfer; in addition the lack of *in vitro* and *in vivo* methods to study parasite viability has limited the development of effective control strategies for this pathogen (Ortega & Sanchez, 2010). While some key aspects of the organism's biology have yet to be fully elucidated, most cases of cyclosporiasis have been associated with imported produce that likely came into contact with contaminated water (Varma et al., 2003). In 2018, the first documented domestic *C. cayetanensis* outbreak was linked to produce grown in the continental United States (Gottlieb et al., 2018). Outbreaks of domestically acquired cyclosporiasis occurred in 2019 and in 2020 (CDCa, 2019; CDCa, 2020). Previously outbreaks had often been associated with produce imported from countries endemic for this organism. This recent finding suggests that *Cyclospora* may now be present in U.S. watersheds (Casillas *et al.,* 2019; Barratt *et al.,* 2021; Mathison *et al.,* 2021). As the frequency of outbreaks and number of illnesses observed continue to drastically increase so does the need for basic research to address knowledge gaps.

Eimeria is a large genus of protozoa that infect the digestive tracts of herbivores or carnivores causing diarrhea, which can be economically important depending on the animal. Oocysts excreted through host feces must undergo internal sporulation before they become infective. This sporulation stage occurs in the

environment and requires ~7-10 days, depending on the species and climactic conditions. Through the fecal-oral route, new hosts can become infected when ingesting sporulated oocysts contaminating food or water. This project includes the use of *Eimeria tenella* as surrogates for *Cyclospora*. The primary host species of these Eimeria is the chicken, and specifically the site of infection is the intestine and the caecum. Coccidiosis causes decreased chicken growth rates, increase in percentage of sick birds and high mortality. Coccidiosis can be economically devastating to the commercial poultry industry (da Cunha et al., 2020).

Cyclospora cayetanensis is a protozoan parasite that causes gastrointestinal illness following consumption, which often occurs with contaminated water or fresh produce. Previous foodborne outbreaks have involved contaminated herbs like basil and cilantro, leafy greens, sugar snap peas, and raspberries and strawberries (Hadjilouka and Tsaltas, 2020). While it is speculated that contaminated water may play a role in transmission, this has not been confirmed. *Cyclospora cayetanensis* is not a zoonotic pathogen. *Cyclospora* is still considered an emerging pathogen by many, due to the limited understanding surrounding the transmission and survival of oocysts in the environment along with difficulties and limitations in detection of oocysts. Over the past two decades there has been an increasing number of illnesses; some associated with fresh produce and some not connected to a specific food. The increase in clinical illness detection could be in part due to an increase in use of rapid culture-independent diagnostic testing. *Cyclospora* is an apicomplexan coccidian protozoan that belongs to the subphylum subphylum Apicomplexa, subclass Coccidiasina, and family Eimeriidae.

At this time there are nineteen *Cyclospora* species that are known to be associated with illness in various animals and only one species that infects humans, *C. cayetanensis* (Ortega and Sanchez, 2010; Lainson, R. 2005). The elucidation of *C. cayetanensis* has included continuing phylogenetic analysis since 1986 (Hadjilouka and Tsaltas, 2020). Phylogenetic analysis of *C. cayetanensis* small subunit rRNA gene sequences, the mitochondrial genome, and apicolast genomes show close relationship to organisms of the *Eimeria* genus as well as a strong relation to *Toxoplasma* (Cinar et al., 2015; Zhao et al., 2013).

There is a significant need for investigation into risk reduction strategies for foodborne protozoa. Global trends indicate an increase in foodborne parasitic infections which affect public health within the United States and internationally. Food safety controls throughout the farm to fork continuum must include a focus on protozoa contamination as well as contamination from bacteria and viruses.

Throughout the scientific literature there is a lack of validated molecular detection approaches for *Cyclospora* in environmental samples. With growing numbers of cases of domestically acquired cyclosporiasis, the need for accurate detection methods is urgent. Although a variety of DNA extraction methods have proven effective for molecular detection of *Cyclospora* oocysts isolated from food samples, the efficiency of DNA extraction has been conservatively estimated at 50% because extraction from oocysts is generally viewed as an inefficient process (Murphy et al., 2018a). Current detection strategies, such as nested PCR assays targeting a segment of 18S rDNA gene have been widely used for molecular detection of

Cyclospora (Kitajima et al., 2014); however, these assays may detect other *Cyclospora* spp. as well as closely related *Eimeria* spp.. Due to these facts, sequencing must be combined with qPCR for confirmation of detection, further highlighting the need for tools with enhanced sensitivity and precision. Discrepancies in the current molecular methods, such as those identified in this study highlight concerns of the produce industry and growers in use and application of varying methods for risk identification.

This project addressed critical data gaps that affect the produce industry across multiple commodities. As the number of *C. cayetanensis* outbreaks associated with produce continue to increase, knowledge of the organism and use of intervention strategies will be crucial to ensure the safety of produce. The first objective was to provide an understanding of the impact of *C. cayetanensis* on waters in the Mid-Atlantic region of the United States, a previously unstudied area. The water sources tested include those that could be used for irrigation of raw agricultural commodities such as surface water (pond, river), reclaimed wastewater, and reclaimed produce wash water. Previous data suggested a number of samples were presumptive positive for *C. cayetanensis* as determined by PCR (Craighead *et al.,* 2019). This project included further analysis of these samples by amplicon (PCR product) sequencing and subsequent analysis.

2.3 Methods

2.3.1 Sample Collection

Samples were previously collected as part of the CONSERVE project and were processed by filtration according to a modified EPA Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration. Several studies have found that the EPA methods can be used not only for *Cryptosporidium* and *Giardia* spp. but also for other protozoan pathogens such as *Cyclospora* and *Toxoplasma.* Immunomagnetic separation, IMS, and immunofluorescence assay, FA, protocols have not been validated for isolation of *C. cayetanensis* from water. The Food and Drug Administration (FDA) validated a qPCR assay for the detection of *C. cayetanensis* on fresh produce that can also be used for the detection of *Cyclospora* in water (Murphy et al., 2018b). The preliminary data was obtained utilizing a combination of the modified EPA 1623 and FDA methods.

Water samples were collected regularly from various sites in Delaware and Maryland over the course of 17 months (June 2017 – October 2018). Seventy-two water samples were collected in total. Approximately 20 L of water was collected and filtered from each site. Water types included surface (pond and tidal river water) and reclaimed (vegetable processing water and treated wastewater) water. A ProDSS water quality water meter (YSI Inc., Yellow Springs, OH) was used to collect information pertaining to water samples' barometric pressure, conductivity, dissolved oxygen, oxygen reduction potential (ORP), pH, nitrate and chlorine levels, salinity,

water temperature and turbidity. Observational information related to sampling such as time of day, cloud cover, recent precipitation (in past 24 hours, 7 days, 14 days), shore condition (onshore vegetation, bank condition), ambient temperature, and tide (if applicable) was also collected.

2.3.2 Filtration, Concentration and Detection

Filtration and concentration of water samples were performed in accordance with the 1623 method. DNA was extracted from water samples using the DNeasy PowerWater Kit (Qiagen, Hilden, Germany), which was developed to isolate DNA from water containing high levels of contaminants making it uniquely suited for extracting DNA from non-traditional agricultural water samples. Pellets equal to or less than 0.5 mL in volume were stored at -80°C until DNA was extracted. Pellets greater than 0.5 mL were subsampled into quantities of 0.5 mL or less and then stored at -80°C. The presumptive presence of *C. cayetanensis,* was determined using quantitative PCR with the QuantiNova Probe PCR Kit (Qiagen, Hilden, Germany). PCR detection of *C. cayetanensis* included use of forward primer (5'- TAG TAA CCG AAC GGA TCG CAT T-3'), reverse primer (5'-AAT GCC ACG GTA GGC CAA TA-3'), and probe (5'-/56-FAM/CCG GCG ATA/ZEN/GAT CAT TCA AGT TTC TAG C/3IABkFQ/-3') (FDA BAM Chapter 19b; Murphy et al., 2017). A standard curve was generated in duplicate for each qPCR run. Protozoan DNA specific to the target organisms were used as a positive control (purchased from ATCC), in addition

to the internal amplification control to test for inhibition. Nuclease-free water and filter eluate were used as negative controls.

To complete the objective of confirming the presence of *C. cayetanensis* in those presumptive positive environmental samples, nested PCR and DNA sequencing were performed.

Table 2: DNA Primer Sequences for Cyclospora-specific PCR Amplification (18S rRNA gene) (Orlandi et al., 2004).

Primer Code	Primer Specificity	Primer Sequence (5'-3')	Amplicon Size (bp)	Application
F1E (Forward)	*Cyclospora* and *Eimeria* spp.	TACCCAATGAAAACAGTTT	636	Primary Amplification
R2B (Reverse)		CAGGAGAAGCCAAGGTAGG		
F3E (Forward)	*Cyclospora* and *Eimeria* spp.	CCTTCCGCGCTTCGCTGCGT	294	Nested Amplification
R4B (Reverse)		CGTCTTCAAACCCCCTACTG		

2.3.3 Sequencing and Phylogenetic Tree Analysis

As detailed above, environmental water samples collected from the Mid-Atlantic region were collected in accordance with a modified EPA 1623.1, "*Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA," method. The method was modified to exclude immunomagnetic separation (IMS) and fluorescent antibody (FA) testing as there are not widely accepted IMS and FA methods for *C. cayetanensis*. The confirmation of the presence of *C. cayetanensis* in presumptive

positive environmental water samples was performed using the 19a:9, "PCR Analysis" method of nested PCR from the U.S. Food and Drug Administration Bacteriological Analytical Manual (Orlandi *et al.,* 2004).

Samples were then purified using the Thermo Scientific GeneJet PCR Purification Kit (Thermo Scientific, Waltham, MA). The purified samples were submitted to the Delaware Biotechnology Institute (Newark, DE) for dideoxy Sanger sequencing with the ABI Prism 3130XL Genetic Analyzer (Applied Biosystems). Data analysis was provided by the University of Delaware Bioinformatics Center. Chromatograms were imported into Geneious (ver 10.2.6). Sequences were trimmed for quality (Q>0.95) and to remove primer sequences, and read pairs were assembled into one contiguous sequence per sample. Contigs were aligned using the SINA aligner (v 1.2.11) with the SILVA global SSU rRNA alignment template using default settings (Pruesse et al. 2012). Aligned sequences were to a 341-position region shared by 30 samples and a tree was constructed using FastTree (v2.1.11) using a GTR model (Price *et al.* 2010). Additional homology searches were performed using BLASTN against the GenBank nucleotide database (Altschul *et al.,* 1990).

2.4 Results

Nested PCR amplification was performed on all presumptive positive water samples. Primers were tested using DNA from *C. cayetanensis*, *E. acervulina* (*Eimeria*), *E. maxima*, and *E. tenella* as positive controls. Nuclease-free water, and

DNA from *C. parvum* (*Cryptosporidium*) served as negative controls. Negative water samples were spiked to test for inhibitors and other factors that could interfere with PCR. Results indicated that the nested PCR method increased sensitivity of the detection. The limit of detection for *C. cayetanensis* decreased from 250 genomic units to 2.5 genomic units.

Forty-two amplicons were sent for sequence analysis. The repeated primary amplicon was used in sequence analysis and sequences compared to those in the GenBank database. Sequence analysis was not straightforward. There were strong base signals at most positions, but a significant number of positions showed a mixed signaling. In these cases, there may be multiple copies of the gene, and this occurs with protozoa due to the presence of multiple life stages within an oocyst, for example. In several samples, there appear to be more than one signal in the sample, so in these cases it is consistent with having mixed amplicons in the sample. In others (like 5.3) the reverse primer is encountered much earlier than expected (i.e., the amplicon is not the correct length).

Based on initial analysis, one sample showed 99.01% similarity to *C. cayetanensis* based on similarity of the sequence in GenBank with 5% query cover. Within the query coverage 80 out of 81 base pairs were match to *C. cayetanensis*. This sample is particularly interesting because it shows that other mixed samples have the potential to contain *C. cayetanensis*, even though it is not the dominant species in the sample. Due to the low query cover, low statistical power and remaining ambiguous

base pairs in this sample, it does not appear on the constructed phylogenetic tree from these environmental water samples.

Several phylogenetic trees were created to better understand the similarities across these samples including reference sequencing that best matched similar microbes from the GenBank database in a blast sequence alignment. A phylogenetic tree is a hypothesis of the evolutionary inheritance of genes across individual taxa. Trees have been used to summarize an organism's pedigree. The organisms are then grouped by similarities in genetic sequence. In terms of sample analysis, several of the samples fall into specific clades, which means that these samples contain organisms are all the descendants of a common ancestor. The phylogenetic tree included here shows the evolutional distance of these organisms (Figure 1). There is greatest similarity in the *Cyclospora* gene fragment (indicated as tCL) with several *Eimeria* and *Isospora* species. *Toxoplasma gondii* is included in this tree as an out-group, so one can see the distance from this organism, which is not as closely related as the others and has low to no similarity with the samples. The outgroup is used as a distant genetic reference.

The phylogenetic tree shows a clade including samples 8.1, 8.2, 8.3, *E. reichenowi* and *E. gruis*. After investigation of members of this clade, it was found that *E. reichenowi* and *E. gruis* causes coccidiosis in wild cranes (Honma et al., 2011; Matsubayahi, 2005). There are two types of cranes that are native to North America: sandhill crane (*Grus canadensis)* and whooping crane (*Grus americana*) (Novilla and

Carpenter, 2004). Given the water types and location that samples 8.1, 8.2, and 8.3 were collected, there is a high likelihood that a crane species could be present and shedding *E. reichenowi* or *E. gruis*.

Within this phylogenetic tree, there is a clade with several samples as well as GenBank reference strains from *I. gryphoni, E. catronensis, I. serinuse,* and *E. lancasterensis.* Interestingly, this clade shows two difference genera sharing the same node, therefore, these samples and reference strains show large genetic similarity. *Isopora gryphoni* had been identified from avian species, specifically a yellow-throated miner bird (*Manorina flavigula*) (Yang et al., 2016) and a goldfinch (*Carduelis tristis*) (Olson et al., 1998). *Eimeria catronensis* has been isolated from a species of bat (Scott and Duszynski, 1997). *Isospora serinuse* has been identified from a canary (*Serinus canaria*) (Yang et al., 2015). *Eimeria Iancasterensis* was first identified with the animal origin of a fox squirrel (*Sciurus niger*) (Motriuk-Smith et al., 2009). Avian species and rodent species are common for the sampling area in Maryland and Delaware.

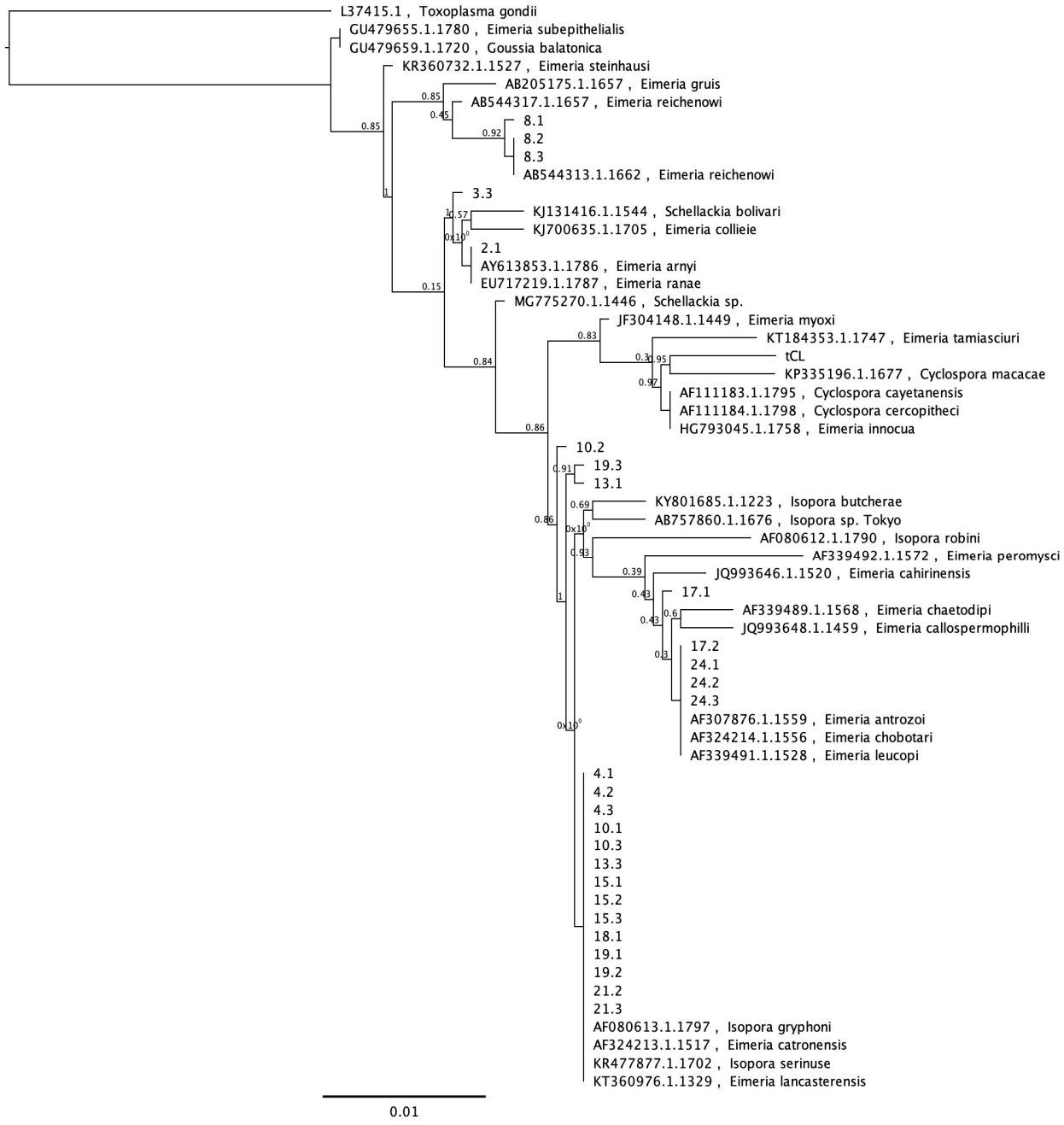
L37415.1 , Toxoplasma gondii
GU479655.1.1780 , Eimeria subepithelialis
GU479659.1.1720 , Goussia balatonica
KR360732.1.1527 , Eimeria steinhausi
AB205175.1.1657 , Eimeria gruis
AB544317.1.1657 , Eimeria reichenowi
8.1
8.2
8.3
AB544313.1.1662 , Eimeria reichenowi
3.3
KJ131416.1.1544 , Schellackia bolivari
KJ700635.1.1705 , Eimeria collieie
2.1
AY613853.1.1786 , Eimeria arnyi
EU717219.1.1787 , Eimeria ranae
MG775270.1.1446 , Schellackia sp.
JF304148.1.1449 , Eimeria myoxi
KT184353.1.1747 , Eimeria tamiasciuri
tCL
KP335196.1.1677 , Cyclospora macacae
AF111183.1.1795 , Cyclospora cayetanensis
AF111184.1.1798 , Cyclospora cercopitheci
HG793045.1.1758 , Eimeria innocua
10.2
19.3
13.1
KY801685.1.1223 , Isopora butcherae
AB757860.1.1676 , Isopora sp. Tokyo
AF080612.1.1790 , Isopora robini
AF339492.1.1572 , Eimeria peromysci
JQ993646.1.1520 , Eimeria cahirinensis
17.1
AF339489.1.1568 , Eimeria chaetodipi
JQ993648.1.1459 , Eimeria callospermophilli
17.2
24.1
24.2
24.3
AF307876.1.1559 , Eimeria antrozoi
AF324214.1.1556 , Eimeria chobotari
AF339491.1.1528 , Eimeria leucopi
4.1
4.2
4.3
10.1
10.3
13.3
15.1
15.2
15.3
18.1
19.1
19.2
21.2
21.3
AF080613.1.1797 , Isopora gryphoni
AF324213.1.1517 , Eimeria catronensis
KR477877.1.1702 , Isopora serinuse
KT360976.1.1329 , Eimeria lancasterensis
0.01

Figure 1. Phylogenetic tree representing NCBI blast results showing genetic similarities to presumptive positive environmental water samples identified as *C. cayetanensis* through nested PCR (numerical entries) with *Toxoplasma gondii* included as an out-group. The sample tCL is the control gene fragment of *C. cayetanensis* purchased from IDT (HMgBlock135m).

2.5 Conclusion

Environmental samples provide great challenges to microbiologists in terms of their microbial content along with potential for inhibitors. In this case, it is not the inhibitors but the complexity of detection of protozoa with numerous life stages and potentially numerous gene copies with these complex environmental samples that provide a challenge. One outcome of this project is informing the need to on perform sequence analysis of these environmental samples given that a positive PCR is likely just a presumptive positive. However, sequence analysis is accompanied by a set of challenges that must be further investigated. Perhaps this project is nearly the tip of the iceberg in terms of elucidating the need to better understand detection *Cyclospora cayetanensis*.

Cluster formation with *E. gruis* and *E. reichenowi* were also found to be constructed in the same way in Matsubayashi's (2005) paper that shows phylogenetic analysis of several *Eimeria* species based on 18 rRNA gene region.

The finding in this dataset that this apicomplexan group of protozoa is difficult to speciate is supported by the literature. The mitochondrial genome of *C. cayetanensis* contains three protein coding genes, cytochrome (*cytb*), cytochrome *C*

oxidase subunit 1 (*cox1*) and cytochrome *C* oxidase subunit 3 (*cox3*), along with 14 large subunits (LSU) and nine small subunit (SSU) fragment rRNA genes (Cinar et al., 2015). "Regions homologous to the *C. cayetanensis* mitochondrial genome 5'-terminus are found in all eimeriid mitochondrial genomes available and suggest this may be the ancestral start of eimeriid mitochondrial genomes (Ogedengbe et al., 2015)." It may be possible that a combination of 18S and COX1 mitochondrial genes could be useful, as used by other scientists, or if there is a means of constructing a longer amplicon for this analysis in order to be more specifically targeting *C. cayetanensis*.

Genetic similarities and phylogenetic analysis are limited to the quality and quantity of published sequences within GenBank. There is limited information about the *Eimeria* and *Isospora* species that were found. As the GenBank database grows with information and sequences of protozoans, stronger genetic references can be made with higher statistical power.

Phylogenetic analysis of apicomplexan protozoa is complex. The need for additional analysis following PCR detection of presumptive *Cyclospora* positive environmental samples is clear; however, the pathway to further identification of these samples remains murky. Computational biologists may be able to clarify this issue given more time for analysis. Microbiologists perform multiple steps of analysis on bacterial detection and confirmation from environmental samples, including biochemical tests, culture isolation, and sequencing. It seems that a similar set of multiple steps to analysis is warranted for these complex environmental samples that

may be positive for *Cyclospora*. Alternate means of detection, using multiple primer sets and potential for inclusion of mitochondrial genomes should also be considered.

REFERENCES

Altschul, S F et al. “Basic local alignment search tool.” *Journal of molecular biology* vol. 215,3 (1990): 403-10. doi:10.1016/S0022-2836(05)80360-2

Barratt, Joel et al. “Investigation of US *Cyclospora cayetanensis* outbreaks in 2019 and evaluation of an improved *Cyclospora* genotyping system against 2019 cyclosporiasis outbreak clusters.” *Epidemiology and infection* vol. 149 e214. 13 Sep. 2021, doi:10.1017/S0950268821002090

Casillas, Shannon M et al. “Cyclosporiasis Surveillance - United States, 2011-2015.” *Morbidity and mortality weekly report. Surveillance summaries (Washington, D.C. : 2002)* vol. 68,3 1-16. 19 Apr. 2019, doi:10.15585/mmwr.ss6803a1

Center for Disease Control and Prevention. (2020a). Cyclosporiasis Outbreak Investigations - United States, 2020. Retrieved December 01, 2020, from https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/2020/seasonal/index.html

Centers of Disease Control and Prevention. (2019a). Cyclosporiasis Outbreak Investigations - United States, 2019. Retrieved December 20, 2020, from https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/2019/a-050119/index.html

Cinar, Hediye Nese et al. “The Complete Mitochondrial Genome of the Foodborne Parasitic Pathogen Cyclospora cayetanensis.” *PloS one* vol. 10,6 e0128645. 4 Jun. 2015, doi:10.1371/journal.pone.0128645

Craighead et al., “The Presence of *Cryptosporidium* spp., *Cyclospora cayetanensis, Toxoplasma gondii,* and *Giardia intestinalis* in Potential Alternative Sources of Agricultural Water: A Conserve Study.” *International Assocaition of Food Protection*, P2-89, 2019.

da Cunha, A. F., Santin, E., & Kogut, M. (2020). Editorial: Poultry Coccidiosis: Strategies to Understand and Control. *Frontiers in veterinary science*, *7*, 599322. https://doi.org/10.3389/fvets.2020.599322

Gottlieb, S. (2018, September 18). Statement from FDA Commissioner Scott Gottlieb, M.D., on the FDA's ongoing efforts to prevent foodborne outbreaks of Cyclospora. Retrieved April 01, 2019, from https://www.fda.gov/news-events/press-announcements/statement-fda-commissioner-scott-gottlieb-md-fdas-ongoing-efforts-prevent-foodborne-outbreaks

Hadjilouka, Agni, and Dimitris Tsaltas. “Cyclospora Cayetanensis-Major Outbreaks from Ready to Eat Fresh Fruits and Vegetables.” *Foods (Basel, Switzerland)* vol. 9,11 1703. 20 Nov. 2020, doi:10.3390/foods9111703

Honma, Hajime et al. “Accurate analysis of prevalence of coccidiosis in individually identified wild cranes in inhabiting and migrating populations in Japan.” Environmental microbiology vol. 13,11 (2011): 2876-87. doi:10.1111/j.1462-2920.2011.02563.x

Kitajima, M., Haramoto, E., Iker, B. C., & Gerba, C. P. (2014). Occurrence of Cryptosporidium, Giardia, and Cyclospora in influent and effluent water at wastewater treatment plants in Arizona. *The Science of the total environment*, *484*, 129–136. https://doi.org/10.1016/j.scitotenv.2014.03.036

Lainson, Ralph. “The genus Cyclospora (Apicomplexa: Eimeriidae), with a description of Cyclospora schneideri n.sp. in the snake Anilius scytale scytale (Aniliidae) from Amazonian Brazil--a review.” *Memorias do Instituto Oswaldo Cruz* vol. 100,2 (2005): 103-10. doi:10.1590/s0074-02762005000200001

Mathison, Blaine A, and Bobbi S Pritt. “Cyclosporiasis-Updates on Clinical Presentation, Pathology, Clinical Diagnosis, and Treatment.” *Microorganisms* vol. 9,9 1863. 2 Sep. 2021, doi:10.3390/microorganisms9091863

Matsubayashi, M., Takami, K., Abe, N. et al. Molecular characterization of crane Coccidia, Eimeria gruis and E. reichenowi, found in feces of migratory cranes. Parasitol Res 97, 80–83 (2005). https://doi.org/10.1007/s00436-005-1404-9

Matsubayashi, M., Takami, K., Abe, N. et al. Molecular characterization of crane Coccidia, Eimeria gruis and E. reichenowi, found in feces of migratory cranes. Parasitol Res 97, 80–83 (2005). https://doi.org/10.1007/s00436-005-1404-9

Meliton N. Novilla & James W. Carpenter (2004) Pathology and pathogenesis of disseminated visceral coccidiosis in cranes, Avian Pathology, 33:3, 275-280, DOI: 10.1080/0307945042000203371

Motriuk-Smith, Dagmara et al. “Species of Eimeria (Apicomplexa: Eimeriidae) from tree squirrels (Sciurus niger) (Rodentia: Sciuridae) and analysis of the ITS1, ITS2, and 5.8S rDNA.” The Journal of parasitology vol. 95,1 (2009): 191-7. doi:10.1645/GE-1653.1

Murphy H. R., Almeria S., da Silva A. J. (2018b). BAM 19b: Molecular Detection of Cyclospora cayetanensis in Fresh Produce Using Real-Time PCR. Available online at: https://www.fda.gov/food/laboratory-methods-food-safety/bam-19b-molecular-detection-Cyclospora-cayetanensis-fresh-produce-using-real-time-pcr (accessed May 20, 2020).

Murphy HR, Almeria S, da Silva AJ. 2017. Molecular detection of *Cyclospora cayetanensis* in fresh produce using real-time PCR *In* Bacteriological analytical manual. U.S. Food and Drug Administration, Silver Spring, MD.

Murphy, H., Nese H., Gopinath, G., Noe, K. E., Chatman, L. D., Miranda, N. E., Da Silva, A. J. (2018a). Interlaboratory validation of an improved method for detection of *Cyclospora cayetanensis* in produce using a real-time PCR assay. *Food Microbiology*, *69*, 170–178. http://doi.org/10.1016/j.fm.2017.08.008

Ogedengbe, Mosun E et al. "A linear mitochondrial genome of Cyclospora cayetanensis (Eimeriidae, Eucoccidiorida, Coccidiasina, Apicomplexa) suggests the ancestral start position within mitochondrial genomes of eimeriid coccidia." International journal for parasitology vol. 45,6 (2015): 361-5. doi:10.1016/j.ijpara.2015.02.006

Olson, V A et al. "A new Isospora sp. from Carduelis tristis (Aves: Fringillidae) from Ontario, Canada." The Journal of parasitology vol. 84,1 (1998): 153-6.

Orlandi, P. A., Frazar, C., Carter, L., Chu D. T. (2004). BAM Chapter 19a: Detection of Cyclospora and Cryptosporidium from Fresh Produce: Isolation and Identification by Polymerase Chain Reation and Microscopic analysis. Available online at: https://www.fda.gov/food/laboratory-methods-food/bam-chapter-19a-detection-cyclospora-and-cryptosporidium-fresh-produce-isolation-and-identification (accessed April 20, 2020).

Ortega, Y. R., & Sanchez, R. (2010). Update on Cyclospora cayetanensis, a Food-Borne and Waterborne Parasite. *Clinical Microbiology Reviews*, *23*(1), 218–234. http://doi.org/10.1128/CMR.00026-09

Scott, D T, and D W Duszynski. "Eimeria from bats of the world: two new species from Myotis spp. (Chiroptera: Vespertilionidae)." The Journal of parasitology vol. 83,3 (1997): 495-501.

Varma, Manju et al. "Detection of Cyclospora cayetanensis using a quantitative real-time PCR assay." *Journal of microbiological methods* vol. 53,1 (2003): 27-36. doi:10.1016/s0167-7012(02)00209-9

Yang, Rongchang et al. “Isospora serinuse n. sp. (Apicomplexa: Eimeriidae) from a domestic canary (Serinus canaria forma domestica) (Passeriformes: Fringillidae) in Western Australia.” Experimental parasitology vol. 159 (2015): 59-66. doi:10.1016/j.exppara.2015.08.020

Yang, Rongchang et al. “Morphological and molecular characterization of Isospora manorinae n. sp. in a yellow-throated miner (Manorina flavigula wayensis) (Gould, 1840).” Experimental parasitology vol. 163 (2016): 16-23. doi:10.1016/j.exppara.2016.01.013

Zhou, Bian-hua et al. “A novel serine/threonine protein phosphatase type 5 from second-generation merozoite of Eimeria tenella is associated with diclazuril-induced apoptosis.” *Parasitology research* vol. 112,4 (2013): 1771-80. doi:10.1007/s00436-013-3336-0

Chapter 3

EVALUATION OF ZERO VALENT IRON (ZVI) AND SAND FILTRATION IN THE REMOVAL AND INACTIVATION OF CRYPTOSPORIDIUM PARVUM AND EIMERIA TENELLA IN ARTIFICAL AGRICULTURAL WATER

3.1 Abstract

Cyclospora cayetanensis is a protozoan parasite that causes gastrointestinal illness following consumption of contaminated produce or water. *Cyclospora* is still considered an emerging pathogen by many, due to the limited understanding and large data gaps surrounding the transmission and survival of oocysts in the environment limitations in detection of oocysts. Previous studies showed genetic relatedness in apicoplast and mitochondrial genomic sequences of *Cyclospora cayetanensis* and *Eimeria* species with 87-92% sequence similarities of the mitochondrial genomes between *C. cayetanensis* and various *Eimeria* species, with the greatest genetic similarity to *Eimeria tenella*. The objective of this study is to determine the efficacy of zero-valent iron (ZVI) filtration in the removal and inactivation of protozoan oocysts from irrigation water to improve pre-harvest food safety and better protect public health. Filtration using the ZVI-sand filtration achieved a 4.30-log reduction of *Cryptosporidium parvum* compared to 1.82 log reduction using sand filtration.

When comparing the two filtration systems, there was a significant difference in efficacy ($p < 0.05$) with ZVI-sand filteration yielding larger reductions of *C. parvum* oocysts. For reduction of *Eimeria tenella* oocysts, ZVI-sand filters achieved a 6-log reduction, whereas sand-only filtration achieved a 2.3-log reduction. There was a significant difference ($p < 0.05$) in ability to inactivate and remove *E.* tenella oocysts, the ZVI-sand filtration performed better compared to the sand alone filtration. The results of this work will facilitate the development of novel on-farm filtration technology and guidelines for commercial applications to control parasitic pathogens in agricultural water, thus improving the safety of produce and control of protozoa in agricultural water. ZVI technology may offer advantages to growers including adaption to existing filtration systems, feasibility, effective filtration, and broad neutralization of numerous biological and chemical hazards with minimal environmental impacts. If the number of *C. cayetanensis* outbreaks associated with produce continues to increase, knowledge of novel intervention strategies will be crucial to enhance produce safety and public health.

Highlights:

- ZVI-sand filtration achieved a 4.30-log genomic copies per mL reduction of *Cryptosporidium parvum* oocysts
- A reduction of 1.82-log genomic copies per mL *C. parvum* oocysts was observed from sand filtration alone

- An observed reduction of 6-log genomic copies per mL was observed for ZVI-sand and 2.3-log f genomic copies per mL or sand filtration for removal of *E. tenella* oocysts.
- ZVI-sand filtration showed a statistically larger parasite reduction then filtration by sand-alone.

Key words: Infectivity, parasite, protozoa, remediation, on-farm technology

3.2 Introduction

Water is a critical agricultural and environmental need which is currently continually being threatened due to water scarcity issues. Agriculture is most demanding for water resources, claiming over 85% of human water consumption (Falkenmark and Rockstrom, 2004). Sustainability of water reuse for agricultural irrigation has become a main concern for ensuring agricultural water security and sustainability for food production. The use of recycled water and nontraditional irrigation water sources, comes with a concern for a potential increased risk in foodborne illnesses. To reduce risk, filtration systems have been studied and implemented to significantly reduce or eliminate pathogens prior to irrigation and contact with the plant surface (Anderson-Coughlin et al., 2021).

Cryptosporidiosis is a disease caused by the protozoan parasite *Cryptosporidium*, where the predominant symptom is watery diarrhea. Illness in humans is predominately associated with infection by *C. parvum* or *C. hominis*, while other species may cause infection particularly in immunocompromised individuals.

Symptoms can be severe or potentially life-threatening for people with weakened immune systems. Symptom onset of cryptosporidiosis is from 2 to 10 days following ingestion of oocysts, with an average of 7 days. The infectious dose for a healthy human ingesting *C. parvum* is between 10 to 100 oocysts and the median infectious dose is 132 oocytes (FDA, 2012; DuPont et al., 1995). Symptoms include watery diarrhea, stomach pain, dehydration, nausea, vomiting, fever, and weight loss. The small intestine is the most common site of infection, however; for immunocompromised persons infection can occur in other areas including the digestive and respiratory tract. Currently the only FDA approved treatment for healthy people of *Cryptosporidium* is nitazoxanide, which is a broad spectrum anti-parasitic used as a deworming agent. However, this treatment is not approved for people one year of age or younger and has not been found effective in immunodeficient patients (Sparks *et al.,* 2015).

The life cycle of *Cryptosporidium parvum* begins with ingestion of sporulated oocysts, each containing 4 sporozoites, which are excreted by the infected host through feces and possibly other routes such as respiratory secretion (CDC, 2015). Transmission of *C. parvum* mainly occurs through contact with contaminated drinking water, recreational water, or food. After ingestion, excystation occurs where the parasite escapes from the oocyst. The sporozoites are then released and parasitize the epithelial cells of the gastrointestinal tract or possibly tissues of the respiratory tract. Asexual and sexual multiplication occurs within the epithelial cells. Fertilization of the macrogamonts by the microgametes occurs which develops oocysts that sporulate in

the infected host. Thick-walled and thin-walled oocysts are produced and infectious upon excretion which allows for fast transmission through the fecal-oral route. At the end of the sexual cycle, 20% of the fully sporulated oocysts are thin-walled oocysts which are excreted within the host and 80% are think-walled oocysts that are excreted into the environment. Thin-walled oocysts cause autoinfection which allows the disease to persist longer in immunocompromised hosts (Leitch and He, 2011).

Based on the Surveillance Overview: National Cryptosporidiosis Case Surveillance 2018 Report, *Cryptosporidium* is the leading cause of U.S. waterborne disease outbreaks (CDC, 2020b; Hlavas et al., 2018) and the third leading cause of U.S. zoonotic enteric illness (Hale et al.,2012). Annually, there are an estimated 748,000 cases of cryptosporidiosis (Scallan et al., 2011). Using data from the Foodborne Diseases Active Surveillance Network (CDC, 2019b), Figure 2 shows the significant increase for the incidence for cryptosporidiosis cases since 2003.

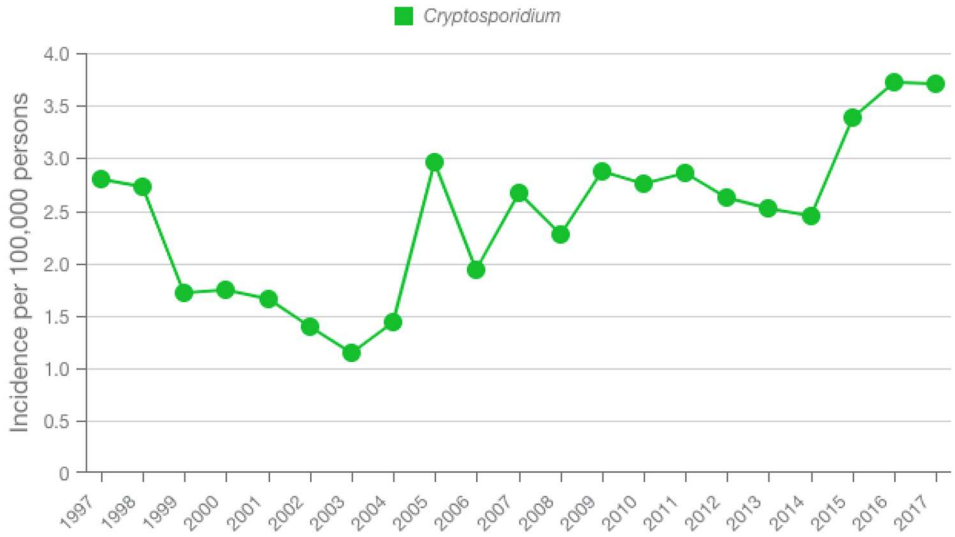

Figure 2: Incidence (Incidence per 100,000 persons) of *Cryptosporidium* Infections by year. Source: FoodNet, Centers for Disease Control and Prevention.

Cyclospora cayetanensis is a critically important waterborne pathogen where data gaps remain regarding infectivity and transmission (Almeria *et al.,* 2019). Given some of the unknowns regarding the study of *Cyclospora*, surrogate protozoa can be useful (Kniel *et al.,* 2007) in evaluating interventions or survival assays. *Eimeria* is a large genus of protozoa, in the same family as *C. cayetanensis*, that infect the digestive tracts of herbivores or carnivores causing diarrhea, which can be economically important depending on the animal. Oocysts excreted through host feces must undergo internal sporulation before they become infective. This sporulation stage occurs in the environment and requires ~7-10 days, depending on the species and climactic conditions. Through the fecal-oral route, new hosts can become infected when ingesting food or water contaminated with sporulated oocysts. This project includes

the use of *Eimeria tenella* as a surrogate for *Cyclospora*. Previous studies have shown the genetic relatedness in apicoplast and mitochondrial genomic sequences of *Cyclospora cayetanensis* and *Eimeria* species. Based on a BLAST search in the NCBI database, the apicoplast sequence of *C. cayetanensis* was most similar to the apicoplast genome of *E. tenella.* BLAST analysis also shows that *C. cayetanensis* has 87-92% sequence similarities to the mitochondrial genomes of various *Eimeria* species (Tang *et al.,* 2015). The primary host species of *E. tenella* is the chicken, and the site of infection is the intestine and the caecum. Coccidiosis causes decreased chicken growth rates, increase in percentage of sick birds and high mortality. Coccidiosis can be economically devastating to the commercial poultry industry (da Cunha et al., 2020).

Given the increased frequency of outbreaks associated with irrigation water (Allende and Monaghan, 2015) and the need for novel mitigation strategies to control protozoa, novel filtration methods continue to be investigated. Zero-valent iron (ZVI) is an effective, non-toxic and low-cost water treatment technology that has the ability to reduce microbiological contaminants from irrigation water. With the diminishing freshwater resources throughout the globe there is an increased demand for alternative water resources of appropriate microbiological quality. Agriculture utilizes approximately 70% of freshwater in the United States (United States Geological Survey, 2016). To address this issue of limited availability and poor irrigation water quality, ZVI technology has been studied to determine the effectiveness for reduction

of microbiological contaminants (Marik *et al.,* 2019; Chopyk *et al.,* 2019; Shearer *et al.,* 2018; Ingram *et al.,* 2012). This research assessed the capability of a ZVI filtration system to remove *C. parvum* and *E. tenella* oocysts. Within the filter, ZVI particles may form iron oxides and other reactive species when reacting with water which may prove to be essential for inactivation of bacterial and viral pathogens and potential inactivation of protozoa after filtration. This reduction process occurs when ZVI reacts with oxygen (O_2), water (H_2O), organic material and minerals (Shi *et al.,* 2011). Previous studies have shown that formation of reactive oxygen species and Fe^{2+} result in significant cell toxicity following use of nanoscale ZVI (Li *et al.,* 2021). Precipitation of both nanoscale ZVI and iron sub-species around and inside the bacterial cell cause denaturation of macromolecules and damage to the intracellular structures ultimately causing cell death (Lefevre *et al.,* 2016).

While the effects of ZVI to reduce bacterial and viral contaminants are known (Marik *et al.,* 2019; Chopyk *et al.,* 2019; Shearer *et al.,* 2018; Ingram *et al.,* 2012); knowledge gaps remain concerning the effect of ZVI filtration on protozoa. Identifying the effectiveness of ZVI to reduce protozoan oocysts from a water source is important for risk reduction of quality irrigation water used on produce and to prevent illnesses to protect public health. The information gathered from this study proved useful in reducing the potential transfer of parasitic contaminants via water to crops that otherwise could beat risk for causing foodborne illness.

3.3 Methods

3.3.1 Parasites

C. parvum (Iowa isolate) oocysts sourced from experimentally infected calves and stored in phosphate-buffered saline (PBS) with penicillin, streptomycin, gentamicin, amphotericin B, and 0.01% Tween 20 were obtained from Waterborne Inc. (New Orleans, LA). The oocysts were stored at 4°C and used within 2 months of the date of shipment, as recommended by Waterborne Inc.

E. tenella (APU-2) (Obtained from Dr. Mark Jenkins, U.S. Department of Agriculture Agricultural Research Service, USDA-ARS) oocysts were propagated every 3-4 months for 3-5 years after initial isolation using standard procedures (Fetterer & Barfield, 2003). *E. tenella* oocyst propagations were checked for purity by microscopy at 400X magnification and confirmed by ITS1-PCR (Jenkins *et al.,* 2006). The oocysts are stored at 4°C in 2% K_2CrO_4 and used within 3 months of propagation.

3.3.2 Column Construction.

Control and test columns were constructed in pairs as previously described (Shearer *et al.,* 2018). A steel mesh (60 mesh; 60 openings per in^2 mesh, wire diameter 0.0075 in.) disk was placed at the bottom of each acrylic column (3.8 inside diameter by 10 cm) and overlaid with a 1-cm layer of Accusand (Covia Holdings, LLC., Independence, OH). For the ZVI test column, a 1:1(w/w) mixture of sand and ZVI (particle size 0.005 to 0.125 in.). These columns were wet packed to the top with

sterile deionized water for the ZVI-sand and sand columns. Columns were sealed with tubing affixed at the center of the top and bottom.

3.3.3 Filtration.

A suspension of 10^6 *C. parvum* or *E. tenella* oocysts (45mL) was pumped over each of two columns (one sand column and one ZVI column) in independent trials and in triplicate. A volume of 3L of sterile water was pumped over the columns at <1mL/min before the inoculation at the start of the filtration trials. The column pore volume was calculated to be 45-ml. Each experiment included the following parts: 1) a 45-mL initial flush with autoclaved pond water, 2) inoculation with a 45-mL parasitic suspension containing one million oocysts (1×10^6) in autoclaved pond water; 3) three individual pulses, of 45-mL each, of autoclaved pond water (3 x 45mL). Liquid was pumped through the columns at a rate of 1 mL/min by using a Tris Peristaltic Pump. Columns were then be flushed with sterile water (500 mL per column). Forty-five 5-mL fractions of eluate were collected in sterile glass tubes with a Foxy Jr. fraction collector. Individual 5-mL samples were stored at 4°C for < 24 h prior to analysis. Eluate samples were analyzed directly by qPCR for *C. parvum* and *E. tenella.* The removal and inactivation of *C. parvum* was also assessed using an integrated cell culture and qPCR method.

3.3.4 Enumeration of *C. parvum* Oocysts and Infectious *Cryptosporidium*.

Prior to inoculation of pond water, *Cryptosporidium* oocysts were bleached, and triple rinsed with HBSS. *Cryptosporidium parvum* positive controls were bleach treated and triple rinsed with Hank's Balanced Salt Solution (HBSS) before application to one well of HCT-8 cells for each trial. Human cell culture was infected with oocysts as described previously (Craighead et al., 2020). The reduction in infectivity was quantified by qPCR. Non-filtered oocysts and nuclease-free water were used as positive and negative controls. The inoculum was applied to the cell monolayers and incubated at 37°C with 5% CO_2 for one hour before removal. The cells were given fresh media and incubated for 24 h. The cells were triple rinsed with Hanks Balanced Salt Solution (HBSS) and detached with 0.5mL of Accustase prior to DNA Extraction with Zymo Research DNA/RNA Extraction Kit. Then qPCR was performed with the Qiagen SYBR Green PCR Kit and Rotor-Gene Q cycler. In addition, each sample was run in duplicate. Quantification of the concentration of oocysts and limit of detection were based in a standard curve generated with serially diluted oocysts. The primer sequences for *Cryptosporidium parvum* detection are as follows: forward primer (CPHSPT2F, 5' TCC TCT GCC GTA CAG GAT CTC TTA 3') and reverse primer (CPHSPT2R, 5' TGC TCT TAC CAG TAC TCT TAT CA 3') (LeChevallier et al., 2003). The PCR reaction volume of 25 µL, included 12.5 µL of Rotor-Gene SYBR Green PCR master mix, 1 µL (400 nM) of each primer, forward and reverse, 8 µL of DNA template, and 2.5 µL of nuclease-free water. PCR was performed using the Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany) with the

following settings: hold at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C or 5 s and combined annealing and extension at 60°C for 10 s.

3.3.5 Enumeration of *Eimeria tenella* Oocysts.

Eimeria tenella oocysts were detected in pooled 5-mL samples of collected filtrate using quantitative PCR analysis. Each sample was tested in duplicate, and all samples were compared to a standard curve using DNA extracted from serially diluted oocysts. A bead beating step was included to break oocyst and sporocyst walls prior to DNA Extraction with the Zymo Research DNA/RNA Extraction Kit. qPCR was performed with the Qiagen SYBR Green PCR Kit and Rotor-Gene Q cycler. Each sample was assessed in duplicate. A standard curve was generated using control oocysts which was then imported and adjusted for each qPCR run. Primers ET_F (TGGAGGGGATTATGAGAGGGA) and ET-R (CAAGCAGCATGTAACGGAGA) were used, according to Thabet et al. (2015). The preparation of the PCR reaction solution consisted of 10 µl of SYBR green master mix (Thermo Scientific, Germany), 0.4 µl of a 25 µM stock of ET-F (final concentration, 0.5µM), 0.4 µM stock of ET-R (final concentration, 0.5 µM), and 7.2 µl nuclease-free water. The template volume was 2 µL, yielding a final 20 µL volume in the reaction tube. Cycling reaction was performed by 40 cycles of 30s at 95ºC, 20s at 62ºC and 20s at 72ºC. A melting curve program involving heating from 60 to 95ºC at a rate of 0.1 ºC/s was performed to create the dissociation curve.

3.3.6 Statistical Analysis.

Data was analyzed using a Matched Pairs test on JMP statistical software. Differences are considered significant with a P value < 0.05.

3.4 Results

3.4.1 Filtration of *Cryptosporidium parvum* Oocysts

Cryptosporidium parvum is a zoonotic pathogen transmitted by oocysts that are spherical in shape and 4-5 microns in size. Filtration through either sand or a 1:1 sand and ZVI mixture, from here on referred to as ZVI:sand, in test columns reduced the initial load of *C. parvum* oocysts (Figure 2) as determined by quantitative PCR. Data are the average of three trials. Filtration using sand alone resulted in an 85.8% reduction of *C. parvum* oocysts across the filtration study, as determined by detection of genomic copies. Filtration using ZVI and sand resulted in a 99.99% reduction of *C. parvum* oocysts as determined by detection of genomic copies over the filtration study. Filtration using the ZVI:sand column achieved a 4.30-log reduction of *C. parvum* compared to a 1.82-log reduction using sand-alone for filtration. There was a statistically significant difference in filtration treatments (p=0.0238) over the course of this study.

Due to the fact that oocysts reproduce in cell culture in an undefined number of asexual reproduction events, it is difficult to state how many oocysts correlate to the detection signal associated with life stages following qPCR and integrated cell culture

(Tandel *et al.,* 2019). This analysis showed that *C. parvum* oocysts remained infectious post-filtration for both treatment columns. Oocysts collected within the first 45 mL were the most infectious, as indicated by a greater signal detected by qPCR. These oocysts had the lowest time in contact with the column, sand or sand:ZVI mix.

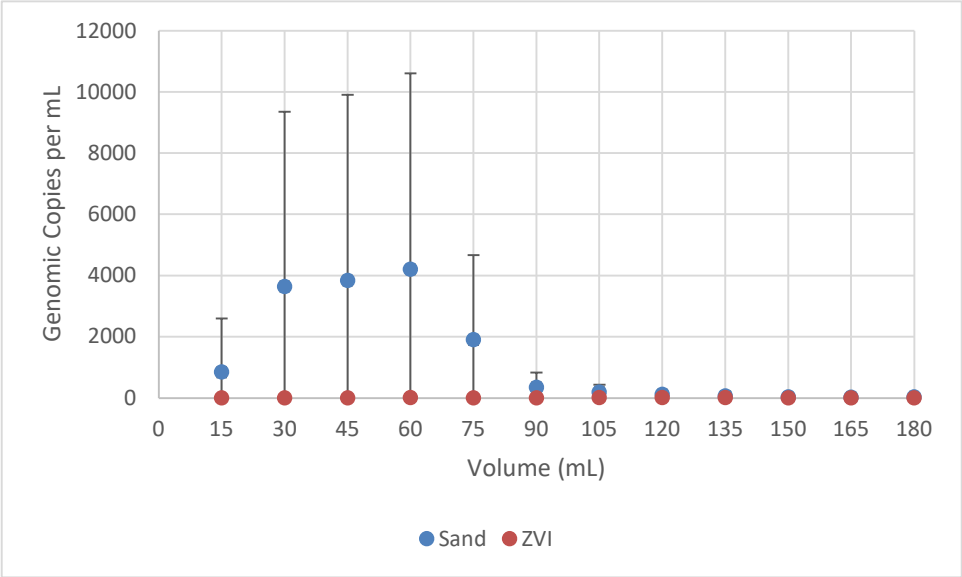

Figure 3. Removal of *Cryptosporidium parvum* oocysts by sand and sand:zvi column filtration. Values shown are indicative of genomic signal from oocysts detected. The lack of signal indicates few to no oocysts detected in those analyzed filtrate samples. Samples of 5 mL each were collected throughout filtration, as follows: the parasitic suspension in samples collected at 0-45 mL, flow through 1 in samples collected at 45-90 mL, flow through 2 in samples collected at 90-135 mL, and flow through 3 in samples collected at 135-180 mL.

3.4.2 Filtration of *Eimeria tenella* Oocysts

Eimeria tenella is an avian pathogen transmitted by oocysts that are ovoid to ellipsoid in shape and approximately 19-22 microns in size. Detection of *E.*

tenella was determined using quantitative PCR from eluate from either sand or a 1:1 sand and ZVI mixed columns. Data are the average of three trials. Filtration with a sand and ZVI mixed column resulted in no detectable *E. tenella* as determined by zero genomic copies per reaction in all samples tested. Filtration using sand alone columns resulted in a 99.5% reduction of *E. tenella*, as determined by analysis of genomic copies across all samples. From total analysis, ZVI:sand filtration achieved a 6-log reduction, whereas sand-only filtration reduced *E. tenella* by-2.3 logs. This is shown in Figure 4.

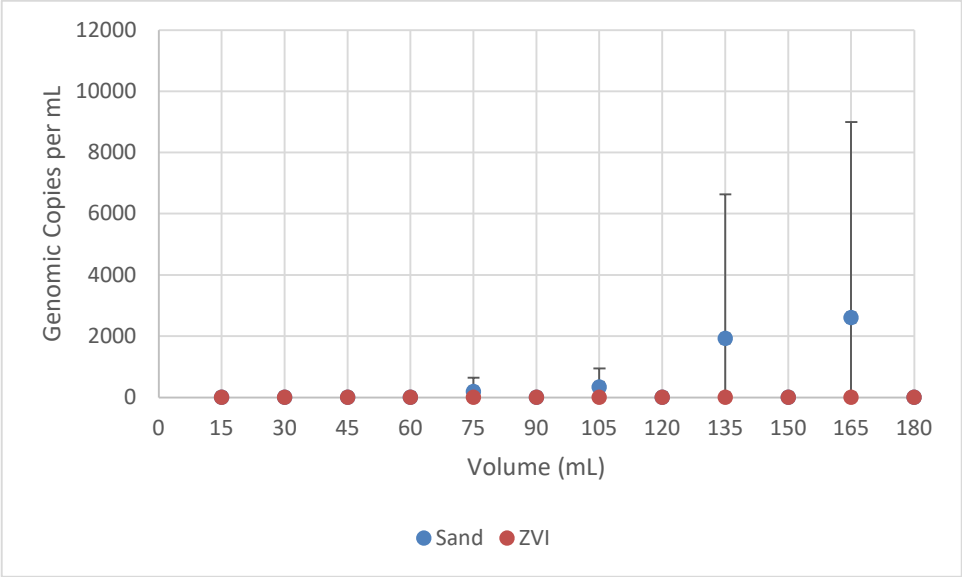

Figure 4. Removal of *Eimeria tenella* oocysts by sand and sand:zvi column filtration. Values shown are indicative of genomic signal from oocysts detected. The lack of signal indicates few to no oocysts detected in those analyzed filtrate samples. Samples of 5 mL each were collected throughout filtration, as follows: the parasitic suspension in samples collected at 0-45 mL, flow through 1 in samples collected at 45-90 mL, flow through 2 in samples collected at 90-135 mL, and flow through 3 in samples collected at 135-180 mL.

3.5 Conclusion

Scarcity of water has caused a demand for non-conventional water sources for agriculture (Eslamian, 2016). Due to the limitations in chemical water treatment systems that can be applied in the field, the development of a novel on-farm filtration system, such as ZVI, along with guidance for commercial applications is essential for the control of parasitic pathogens in agricultural water, thus improving the safety of produce and reducing parasitic foodborne illness. Irrigation water treatment remains a food safety priority. ZVI technology may offer advantages to small and mid-sized growers including adaption to existing filtration systems, feasibility, effective filtration and broad neutralization of numerous biological and chemical hazards with minimal environmental impacts.

Zero-valent iron (ZVI) filtration was proven to reduce *Escherichia coli* and *Listeria monocytogenes* in surface water used for irrigation purposes (Marik et. *al* 2019). Marik (2019) also found that 35%:65% ZVI-sand was not sufficient at inactivating *E. coli*, therefore, *E. coli* may be more resistant to ZVI-sand-mediated killing compared to *L. monocytogenes.* Filters with 35%:65% v/v ZVI-sand were

determined to not be effective at reducing counts of *E. coli*. From the conclusion of this research study, a 50%:50% v/v ZVI-sand was selected for the column construction in the current study.

The reduction of *E. coli* O157:H12 using a ZVI biosand filter and a control (no treatment) for irrigation water which was then applied to spinach plants was determined (Ingram *et al.,* 2012). This study concluded that ZVI biosand filters were more effective in reducing *E. coli* O157:H12 populations in irrigation water compared to the biosand filter. This study used a 1:1 ZVI-Sand ratio by weight which showed a significant lower reduction of. *E. coli* O157:H12 on Day 0 compared to the biosand filters (Ingram *et al.,* 2012). In a similar study, the reduction of *E. coli* in surface water and on leafy greens using a ZVI water filtration system was performed (Kim et al., 2020). This study evaluated a 50%:50% ZVI-Sand filtration system to reduce *E. coli* populations in pond water and the *E. coli* populations were transferred to growing spinach plants in a field trial. This study found that the 50%:50% ZVI-Sand filtration system successfully inactivated *E. coli* population by 70%, whereas the 100% sand filtration system only reduced the *E. coli* population by 10%. In conclusion, this study showed data to prove that ZVI-sand filtration significantly reduced *E. coli* population in water compared to sand fitration (Kim et *al.,* 2020).

In Kim 2021, evaluated ZVI-sand filtration in reducing *E. coli* levels based on influent water type and the percentage of ZVI in sand filters for improving the microbial quality of agricultural water. Filters that were made of 50% ZVI/50% sand

showed did not show a significant difference (p =0.48) in the reduction in *E. coli* in pond water compared to filters made of 35% ZVI/65% sand. This study also found that the water types impact the *E. coli* removal with better reduction in deionized water compared to pond water. This study shows the impact of water types and percentage of ZVI-sand constructed in the filers impact of removal of *E. coli* in relation to overall microbial quality of agriculture water (Kim, *et al.,* 2021

A ZVI filtration system was also assessed for the ability to reduce norovirus surrogates, Murine norovirus and Tulane virus by Shearer (2018); using the system to be tested in this study. A 1:1 mixture of ZVI and sand filtration resulted in a 2-log reduction of Tulane virus and a 3-log reduction of Murine norovirus. The inoculated water was then irrigated on lettuce plants resulting in a 1.5- to 2-log reduction in Tulane virus particles with ZVI filtration compared to sand alone (Shearer et al., 2018). These finding suggest that ZVI is more efficient at reducing norovirus surrogates from irrigation water which will lower illness risk and protect public health.

All of these findings suggest that ZVI water filtration system is efficient at reducing bacterial and viral contamination for use of produce irrigation. In previous studies, ZVI-sand filtration systems were either significantly better at reducing inoculated microorganisms or not significantly different than the sand filtration system in the cause of water inoculated with *Listeria monocytogenes*. Based on the previous research, this research study used a 50%:50% ZVI-sand filtration system. These findings also warrant further investigation into large-scale simulations of water

remediation for bacterial, viral and potentially parasitic contaminants for potential application in the treatment of water used for irrigation, food processing, and drinking.

Data gaps exist in the literature regarding protozoan reduction and inactivation by water filtration systems. An accomplishment of this project is providing evidence that sand filtration provides efficient removal of small and large protozoa and inclusion of ZVI with that sand can increase removal efficiencies of both *Cryptosporidium* and *Eimeria*.

The results of this study prove that ZVI-Sand filters lead to a significantly larger reduction in both *C. parvum* and *E. tenella* compared to sand-only filtration. Filtration using ZVI-sand achieved a 4.30-log reduction compared to 1.82-log reduction using sand-alone for filtration of *C. parvum*. Reductions were similarly seen for *E. tenella,* ZVI-sand filtration achieved a 6-log reduction, whereas sand-only filtration showed reduction by 2.3 logs. Both filtration systems, ZVI-sand and sand alone, were more effective at reduction of *E. tenella* compared to *C. parvum* due to size of the protozoan. *C. parvum* oocysts were shown to be more infectious in the earlier flush volume, due to potential of less contact time.

ZVI filtration systems have been found to reduce a variety of foodborne pathogens. Filtration including ZVI has been shown to reduce *Escherichia coli* and *Listeria monocytogenes* from surface water intended to be used for irrigation and reduced recovery from irrigated lettuce plants (Marik et al., 2019). Successful reductions in norovirus surrogates, murine norovirus and Tulane virus, has been shown using the same ZVI filtration system (Shearer et al., 2018). This study has

added comprehensive evidence that ZVI filtration system used for water intended for irrigation purposes can significantly reduce pathogens better compared to sand alone.

Biosand filters are used for recreational water purification and on some farms. The incorporation of zero-valent iron has previously been shown to increase the removal and inactivation of bacteria and viruses by filtration. These findings suggest that zero-valent iron can enhance the efficacy of filtration for removing protozoa of varying sizes (5-22 microns).

In conclusion, this study demonstrated a greater removal in *C. parvum* and *E. tenella* for the columns containing a mixture of sand and ZVI compared to columns with sand alone. These results warrant further investigation into large-scale water filtration testing for removal of bacteria, viruses and protozoa for the purposes of treating various water types intended to be used for drinking, food processing and agricultural irrigation.

REFERENCES

Allende, A., & Monaghan, J. (2015). Irrigation Water Quality for Leafy Crops: A Perspective of Risks and Potential Solutions. *International journal of environmental research and public health*, *12*(7), 7457–7477. https://doi.org/10.3390/ijerph120707457

Almeria, Sonia et al. "*Cyclospora cayetanensis* and Cyclosporiasis: An Update." *Microorganisms* vol. 7,9 317. 4 Sep. 2019, doi:10.3390/microorganisms7090317

Anderson-Coughlin, Brienna L et al. "Zero-Valent Iron Filtration Reduces Microbial Contaminants in Irrigation Water and Transfer to Raw Agricultural Commodities." *Microorganisms* vol. 9,10 2009. 23 Sep. 2021, doi:10.3390/microorganisms9102009

Centers for Disease Control and Prevention (2019b). FoodNet Fast: Pathogen Surveillance. Retrieved May 20, 2020, from https://wwwn.cdc.gov/foodnetfast/

Centers for Disease Control and Prevention. (2015). Pathogen & Environment, Parasites-Cryptosporidium. Retrieved September 10, 2019, from https://www.cdc.gov/parasites/crypto/pathogen.html

Centers for Disease Control and Prevention. (2020b). Cryptosporidiosis NNDSS Summary Report for 2018. Retrieved May 20, 2020, from https://www.cdc.gov/healthywater/surveillance/cryptosporidium/cryptosporidium-2018.html

Chopyk, Jessica et al. "Zero-valent iron sand filtration reduces concentrations of virus-like particles and modifies virome community composition in reclaimed water used for agricultural irrigation." BMC research notes vol. 12,1 223. 11 Apr. 2019, doi:10.1186/s13104-019-4251-y

Craighead, Shani et al. "Cold Atmospheric Plasma Jet Inactivates Cryptosporidium parvum Oocysts on Cilantro." *Journal of food protection* vol. 83,5 (2020): 794-800. doi:10.4315/0362-028X.JFP-19-442

da Cunha, Anderson Ferreira et al. "Editorial: Poultry Coccidiosis: Strategies to Understand and Control." *Frontiers in veterinary science* vol. 7 599322. 23 Oct. 2020, doi:10.3389/fvets.2020.599322

DuPont, H. L., Chappell, C. L., Sterling, C. R., Okhuysen, P. C., Rose, J. B., & Jakubowski, W. (1995). The infectivity of Cryptosporidium parvum in healthy volunteers. *The New England journal of medicine*, *332*(13), 855–859. https://doi.org/10.1056/NEJM199503303321304

Eslamian, S. (Ed.), Urban Water Reuse Handbook, CRC Press, Boca Ratón (2016)

Falkenmark M., Rockström J., Balancing Water for Humans and Nature: The New Approach in Ecohydrology, (Earthscan, Sterling, VA, 2004).

Fetterer, R H, and R C Barfield. "Characterization of a developmentally regulated oocyst protein from Eimeria tenella." *The Journal of parasitology* vol. 89,3 (2003): 553-64. doi:10.1645/GE-3159

Food and Drug Administration, Department of Health and Human Services (2012). Bad Bug Book, Foodborne Pathogenic Microorganisms and Natural Toxins (Second Edition).

Hale RH, Scallan E, Cronquist AB, et al., Estimates of enteric illness attributable to contact with animals and their environments in the United States, Clinical Infect Dis 2012:54:472–9. DOI: https://doi.org/10.1093/cid/cis051external icon.

Hlavsa MC, Cikesh BL, Roberts VA, et al. Outbreaks associated with treated recreational water — United States, 2000–2014. MMWR Morb Mortal Wkly Rep 2018;67:547–551. DOI: http://dx.doi.org/10.15585/mmwr.mm6719a3

Ingram, D. T., Callahan, M. T., Ferguson, S., Hoover, D. G., Shelton, D. R., Millner, P. D., Sharma, M. (2012). Use of zero-valent iron biosand filters to reduce Escherichia coli O157:H12 in irrigation water applied to spinach plants in a field setting. *Journal of Applied Microbiology*, *112*(3), 551–560. http://doi.org/10.1111/j.1365-2672.2011.05217.x

Jenkins, M C et al. "Improved polymerase chain reaction technique for determining the species composition of Eimeria in poultry litter." *Avian diseases* vol. 50,4 (2006): 632-5. doi:10.1637/7615-042106R.1

Kim, S., Bradshaw, R., Kulkarni, P., Allard, S., Chiu, P. C., Sapkota, A. R., Newall, M. J., Handy, E. T., East, C. L., Kniel, K. E., & Sharma, S. (2020). Zero-Valent Iron-Sand filtration reduces *Escherichia coli* in Surface Water and Leafy Green Growing Environments. *Front. Sustain. Food Syst.* https://doi.org/10.3389/fsufs.2020.00112

Kim S, Eckart K, Sabet S, Chiu PC, Sapkota AR, Handy ET, East CL, Kniel KE, Sharma M. *Escherichia coli* Reduction in Water by Zero-Valent Iron–Sand Filtration Is Based on Water Quality Parameters. *Water*. 2021; 13(19):2702. https://doi.org/10.3390/w13192702

Kniel, Kalmia E et al. "High hydrostatic pressure and UV light treatment of produce contaminated with Eimeria acervulina as a Cyclospora cayetanensis surrogate." *Journal of food protection* vol. 70,12 (2007): 2837-42. doi:10.4315/0362-028x-70.12.2837

LeChevallier, Mark W et al. "The potential for health risks from intrusion of contaminants into the distribution system from pressure transients." *Journal of water and health* vol. 1,1 (2003): 3-14.

Lefevre, E., Bossa, N., Wiesner, M. R., & Gunsch, C. K. (2016). A review of the environmental implications of in situ remediation by nanoscale zero valent iron (nZVI): Behavior, transport and impacts on microbial communities. *The Science of the total environment*, *565*, 889–901. https://doi.org/10.1016/j.scitotenv.2016.02.003

Leitch, G. J., & He, Q. (2012). Cryptosporidiosis-an overview. *Journal of biomedical research*, *25*(1), 1–16. https://doi.org/10.1016/S1674-8301(11)60001-8

Li, Yaru et al. "Remediation of soil contaminated with organic compounds by nanoscale zero-valent iron: A review." The Science of the total environment vol. 760 (2021): 143413. doi:10.1016/j.scitotenv.2020.143413

Marik, C.M., Anderson, B., Gartley, S., Craighead, S., Bradshaw, R., Kulkarni, P., Sharma, M., and Kniel, K.E. (2019). The efficacy of zero valent iron-sand filtration on the reduction of E. coli and L. monocytogenes in surface water for use in irrigation. *Environmental Research*, *In press*.

Marik, Claire M et al. "The efficacy of zero valent iron-sand filtration on the reduction of Escherichia coli and Listeria monocytogenes in surface water for use in irrigation." Environmental research vol. 173 (2019): 33-39. doi:10.1016/j.envres.2019.02.028

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Jones, J. L., & Griffin, P. M. (2011). Foodborne illness acquired in the United States--major pathogens. *Emerging infectious diseases*, *17*(1), 7–15. https://doi.org/10.3201/eid1701.p11101

Shearer, A.E.H. and Kniel, K.E. (2018). Enhanced removal of norovirus surrogates, murine norovirus and Tulane virus, from aqueous systems by zero-valent iron. *J. Food Protection*, 81(9), 1432-1438.

Shi, Z., Nurmi, J. T., & Tratnyek, P. G. (2011). Effects of nano zero-valent iron on oxidation-reduction potential. *Environmental science & technology*, *45*(4), 1586–1592. https://doi.org/10.1021/es103185t

Sparks, Hayley et al. "Treatment of Cryptosporidium: What We Know, Gaps, and the Way Forward." Current tropical medicine reports vol. 2,3 (2015): 181-187. doi:10.1007/s40475-015-0056-9

Tang, K., Guo, Y., Zhang, L. et al. Genetic similarities between Cyclospora cayetanensis and cecum-infecting avian Eimeria spp. in apicoplast and mitochondrial genomes. Parasites Vectors 8, 358 (2015). https://doi.org/10.1186/s13071-015-0966-3

Thabet, Ahmed et al. "Combination of cell culture and qPCR to assess the efficacy of different anticoccidials on Eimeria tenella sporozoites." *Parasitology research* vol. 114,6 (2015): 2155-63. doi:10.1007/s00436-015-4404-4

United States Geological Survey. (2016). Irrigation Water Use, the USGS Water Science School. Retrieved November 28, 2016, from http://water.usgs.gov/edu/wuir.html

Chapter 4

EXAMINATION OF GASEOUS CHLORINE DIOXIDE AS A SANITIZER TO REDUE CRYPTOSPORIDIUM PARVUM ON PRODUCE

4.1 Abstract

This study investigated the effects of gaseous chlorine dioxide at varying treatment times on *Cryptosporidium parvum* on produce, baby cut carrots and grape tomatoes. Grape tomatoes (approx.40g) and baby-cut carrots (approx. 35g) were spot inoculated (10^6 oocysts) with 100-µl of *C. parvum* suspension in 10-µl droplets randomly distributed on the produce samples. Inoculated produce samples were treated with 50 g of each gaseous chlorine dioxide precursor for 0, 1 and 3 hours in a 35-L enclosed chamber. Significant reductions were shown at both 1 hour and 3-hour treatment times for both produce types. After tomatoes were treated with gaseous chlorine dioxide for one hour, a 4.82-log reduction was shown. After three hours of treatment with gaseous chlorine dioxide, *C. parvum* remained undetectable. For carrots, one hour of gaseous chlorine dioxide treatment resulted in a 3.6-log reduction of *C. parvum.* Carrots that were treated for three hours showed a 5.2-log reduction of *C. parvum.* Water activity was measures for each produce type before and after exposure to treatment. There was no significant change in water activity after 1 hour or 3 hours of treatment with gaseous chlorine dioxide for baby cut carrots or grape tomatoes. Colorimeter data was collected before and after treatment with gaseous chlorine dioxide for examination of food quality. A delta E^*_{ab}, overall color change,

was calculated for each treatment to monitor overall physical change of the produce. Carrots after one-hour of treatment had a delta E^*_{ab} of 14.75 and after three hours of treatment delta E^*_{ab} value of 43.46, therefore a large color change was observed for baby cut carrots. Whereas, delta E^*_{ab}, values for grape tomatoes after 1- and 3-hour treatments were 4.01 and 1.87, respectively, thus a slight color change was observed. This study provides useful information of treatment concentration and times for effective reduction of *C. parvum* on produce and collection of data to examine for maintaining food quality.

4.2 Introduction

Non-thermal methods have been investigated for many years for use on fresh produce, as they maintain organoleptic properties of raw fruits and vegetables. Gaseous chlorine dioxide is one such method that has been evaluated as a bactericide and fungicide on raw produce as an alternative for food sanitizers. This treatment is more advantageous compared to traditional methods of chlorinated rinse water because it can break down phenolic compounds and does not react with ammonia. This treatment also has a high sporicidal activity, its activity is not markedly affected by pH and has 2.5 times the oxidation capacity of chlorine. Gaseous chlorine dioxide is more stable and has a higher generation yield compared to ozone treatment (Han *et al.,* 2004). Thick-walled *Cryptosporidium* oocysts are extremely resistant to environmental conditions which causes high resistance to chlorine disinfection for drinking water and produce wash water. *Cryptosporidium* is one of the most resistant

organisms in water and able to withstand very high chlorine levels for 18 hours with no inactivation observed (Korich et al., 1990). To ensure clean drinking water and produce wash water, alternatives for chlorine disinfectants are needed to ensure inactivation of *Cryptosporidium* is achieved.

Chlorine dioxide (ClO_2) gas has been identified as a promising alternative to aqueous chlorine in the sanitation of raw agricultural commodities (Sy *et al*., 2005; Ortega *et al*., 2008). Gaseous ClO_2 is a strong oxidizing agent with bactericidal, fungicidal, and sporicidal activity and an oxidation capacity 2.5 times that of chlorine (Sy *et al.*, 2005; Chai *et al.*, 2020).

Several studies have shown that gaseous ClO_2 generated from dry media can be effective against a wide variety of foodborne pathogens on produce surfaces. According to Sy (2005), treatment for 30 to 120 mins with 8.0 mg/liter of gaseous ClO_2 significantly reduced *Salmonella* populations on blueberries by 2.4- to 3.7-log CFU/g. It was noted that lethality was higher to cells on the skin of the blueberries compared to the stem scar or calyx. In the same study, the same parameters were applied to strawberries and raspberries inoculated with *Salmonella*. A 3.8 to 4.0-log CFU/g reduction was achieved on strawberries and a 1.5-log CFU/g reduction was achieved on raspberries (Sy *et al.*, 2005). Treatment of tomatoes, blueberries, and baby-cut carrots inoculated with *E.coli*, *Salmonella*, *Listeria monocytogenes* with cumulative gaseous ClO_2 exposures of 300, 1300, and 1600 ppm-h caused > 4-log CFU/g reductions of the pathogens (Chai *et al.*, 2020). Lettuce inoculated with *Escherichia coli* O157:H7, *Listeria monocytogenes,* or *Salmonella* Typhimurium and

treated with ClO2 gas for 30 min had a 3.4-log reduction in *E.coli,* a 4.3-log reduction in *Salmonella* Typhimurium, and a 5.0-log reduction in *L. monocytogenes* when compared with the control. Higher reductions were observed with longer treatment times. After 1 h, the three pathogens were reduced by 4.4, 5.3, and 5.2-log CFU/g, respectively. After 3 h, pathogens were reduced by 6.9, 5.4, and 5.4-log CFU/g, respectively (Lee *et al.*,2004). *Cryptosporidium parvum* oocysts treated for 20 min with ClO_2 at 4.1 mg/liter had a 2.6 and 3.31 MPN log reduction in infectivity on basil and green leaf lettuce, respectively (Ortega *et al*., 2008). Ortega (2008) noted that the same treatment did not impact *Cyclospora cayetanensis* sporulation on basil and green leaf lettuce.

The objective of this study is to examine the efficacy of gaseous chlorine dioxide as a sanitizer for the reduction of *C. parvum* on baby cut carrots and tomatoes. Treatment time of 1 and 3 hours will be assessed for reduction of *C. parvum* and produce quality. Indicators of produce quality include colorimeter data, water activity measurements and chlorine dioxide measurements. The results of this study will aid in the complete understanding of the reduction potential for a variety of foodborne pathogens and have an understanding on the physical changes of product due to the treatment with gaseous chlorine dioxide.

4.3 Methods

4.3.1 Oocysts and Cell Line Preparation.

Live oocysts were purchased from Waterborne Inc. Human adenocarcinoma (HCT-8) cells were grown in RPMI 1640 medium with 2% or 10% fetal bovine serum, 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and antibiotics-antimycotic (100 U/ml of penicillin G sodium, 100 µg/ml of streptomycin sulfate, and 0.25 µg/ml amphotericin B as Funfizone). The cells were incubated at 37°C with 5% CO_2. For routine passage (every 2 or 3 days) 2% or 10% fetal bovine serum were used as the maintenance medium, whereas 10% FBS were used as a growth medium whenever parasites were grown. Forty-eight hours prior to treatment, HCT-8 cells were seeded into sterile tissue culture 6-well chamber slides. Samples were incubated at 37°C with 5% CO_2. Once confluency of cells was achieved, monolayers were incubated with either treated or untreated oocysts at approximately 10^6 oocysts per well. At 48 hrs post-inoculation, DNA were extracted, and qPCR performed for infectivity. A standard curve were used for comparison.

4.3.2 Produce Tested and Inoculation.

Grape tomatoes and baby-cut carrots were purchased from a local grocery store in Newark, DE and stored at 4°C. Each sample contained three grape tomatoes

(approx. 40g) or two baby-cut carrots (approx. 35g). Samples were placed separately on an open petri plate in preparation for inoculation.

Cherry tomato and baby-cut carrot samples were spot inoculated (10^6 oocysts) with 100 µl of *C. parvum* suspension in 10 µl droplets randomly distributed on the samples. The produce samples were placed inside the biohazard chamber hood for 30 minutes at room temperature. Treatment control samples included inoculated on plastic weigh boats. Samples were then placed in the desiccator for treatment with gaseous chlorine dioxide gas.

4.3.3 Gaseous ClO_2 Treatment System

Treatments were performed in a 35-Liter (35.56(L) x 36.46(W) x 34.59(H) cm^3) polycarbonate vacuum desiccators (Bel-Art, SP Scienceware, PA, USA). The chamber was coated with black acrylic paint to prevent photodegradation due to ClO_2 photosensitivity. Three brushless 12-V DC fans were placed within the chamber to circulate the air connected to a power block. A hygrometer/thermometer recorder were used to monitor humidity and temperature inside the treatment cabinet during treatment (Ortega, 2008). During the treatment, inoculated produce is placed on open petri plates and positive control were inoculated on sterile plastic weigh boats. The plastic breaker was located in the middle, which contains the sachet with a mixture of the two precursors (A, granular porous solid impregnated with sodium chlorite, and B, granular porous solid impregnated with acid and an acid precursor ferric chloride). The generation of gaseous chlorine dioxide was activated by shaking the mixture of

the precursors in the sachet prior to placing the sachet in the plastic beaker. A gaseous ClO_2 detector (PortaSens II D16, Analytical Technology Inc., Collegeville, PA, USA) probe and a thermo-hygrometer (Fisher Science Hygrometer/Thermometer MK 516928, Thermo Fisher Scientific, Waltham, MA, USA) were inserted into the desiccator for real-time measurement of the headspace concentration of ClO_2, temperature, and relative humidity (RH) during treatments.

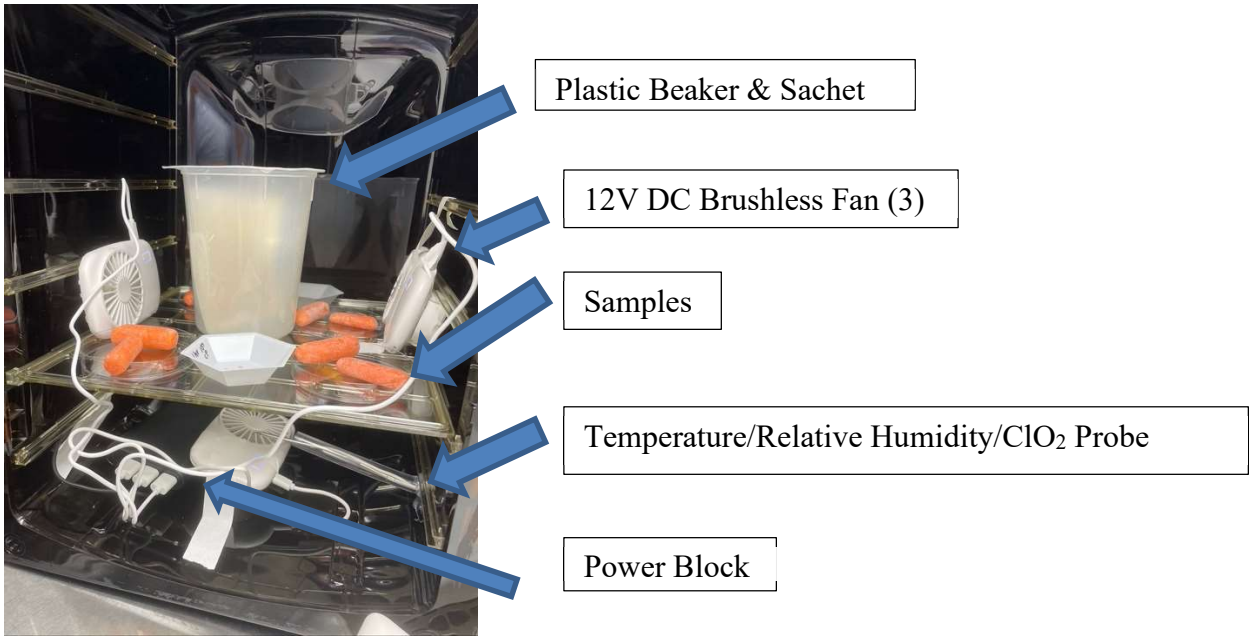

Figure 5. Chamber setup with 3 fans attached to a power block, plastic beaker to hold the sachet with chemical A and B, and samples in open petri plates on the shelf. Temperature, relative humidity, and gaseous chlorine dioxide meter were connected through acrylic tubing for real-time data collection.

4.3.4 Treatment with Gaseous Chlorine Dioxide (ClO_2 Gas).

Treatments were performed as described previously (Kingsley et al., 2018; Rane et al., 2020). Before starting the treatment, 50 g of dry chemical A (granular

porous solid impregnated with sodium chlorite) and 50 g of dry chemical B (granular porous solid impregnated with acid and an acid precursor, ferric chloride) were weighed, mixed and immediately placed inside a sachet provided by the manufacturer. The sachet was placed in the treatment cabinet and treatment time began after desiccator door was sealed. Temperature and relative humidity were determined every 15 minutes. Concentration of gaseous chlorine dioxide (mg/L) was recorded every five minutes throughout the treatment period. Samples were exposed to gaseous chlorine dioxide in the chamber for 1 or 3 hours.

After treatment with gaseous chlorine dioxide, samples were rinsed with 3 ml RPMI 1640 medium with 10% fetal bovine serum (FBS) to remove oocyst from produce samples. The rinse was added to the 6-well plate containing HCT-8 cells. Each sample consisted of two baby-carrots or three grape tomatoes, three produce samples were added to the desiccator per time point. Samples were processed in duplicate in cell culture. The inoculated cells were incubated for 48 h at 37°C and with 5% CO_2 and infection determined as describe previously (Ortega et al., 2008).

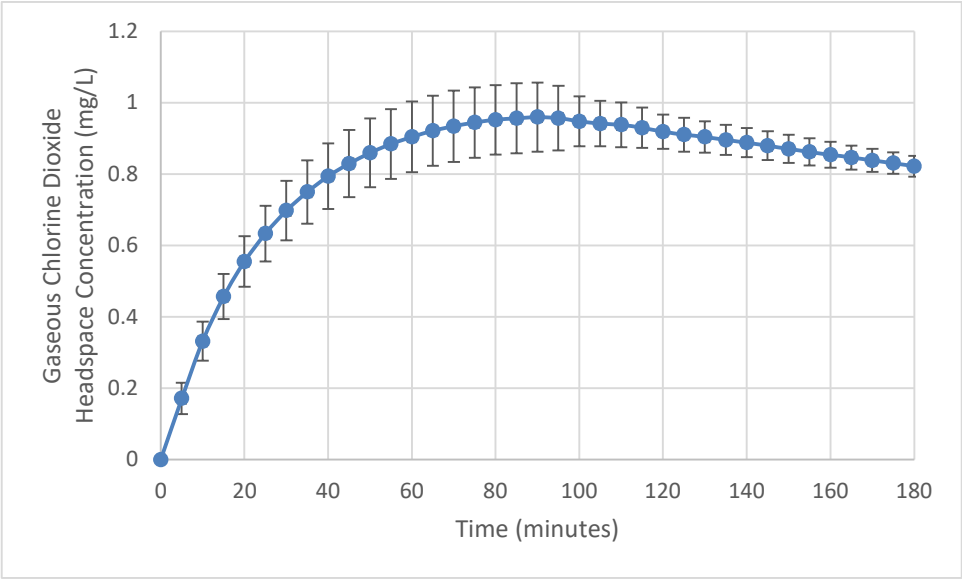

Figure 6. ClO_2 headspace concentration (mg/L) over 3 hours of gaseous chlorine dioxide treatment using 50 g of each precursor. Gaseous chlorine dioxide (mg/L) was measured every five minutes including standard deviations indicated by error bars.

4.3.5 Physical Change Measurements.

Physical changes, color and water activity, from the grape tomatoes and baby-cut carrots were observes at 0, 1 and 3 hours to determine quality effects from exposure to gaseous chlorine dioxide. Colorimeter data (L*, a*, b* values) were collected, using a WR10QC Portable Color Analyzer Digital Precise Colorimeter (Kamifaryaei). The magnitude of color change is expressed as, delta E^*_{ab}, which is defined as $\sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$ (Black *et al.,* 2010). The delta E^*_{ab} value indicates a change but does not indicate the direction of change. Cross sections of grape tomatoes and baby-cut carrots were taken and used to determine water activity

measurements using an AquaLab water activity meter. Six measurements were taken per time point for color and water activity testing.

4.3.6 Chlorine Dioxide Residual Test

After treatment with gaseous chlorine dioxide, samples were tested for residual chlorine dioxide from produce. Samples were taken before treatment, directly after treatment and 24 h post-treatment. Samples were stored at 4°C after treatment for the 24 h post-treatment test for residual chlorine dioxide. Each sample was rinsed 30 times with 5mL of DI water. Samples were tested using the Hach Chlorine Dioxide Color Disc Test Kit.

4.3.7 Enumeration of Infectious *Cryptosporidium*

Human cell culture was infected with oocysts as described previously (Craighead et al., 2020). The reduction in infectivity was quantified by qPCR. *Cryptosporidium* filtrate was bleached, and triple rinsed with PBS before application to 5 wells of HCT-8 cells. Untreated oocysts and nuclease-free water was used as positive and negative controls. The inoculum was applied to the cells and incubated at 37°C with 5% CO_2 for one hour before removal. The cells were given fresh media and incubated for 24 h. The cells were triple rinsed with Hanks Balanced Salt Solution (HBSS) and detached with 0.5mL of Accustase prior to DNA Extraction with the Zymo Research DNA/RNA Extraction Kit. Then qPCR was performed with the Qiagen SYBR Green PCR Kit and Rotor-Gene Q cycler. In addition, each sample was

run through qPCR in duplicate. Quantification of the concentration of oocysts and limit of detection were based in a standard curve generated with serially diluted oocysts. The primer sequences for *Cryptosporidium parvum* detection used were the following: forward primer (CPHSPT2F, 5' TCC TCT GCC GTA CAG GAT CTC TTA 3') and reverse primer (CPHSPT2R, 5' TGC TCT TAC CAG TAC TCT TAT CA 3') (LeChevallier et al., 2003). The PCR reaction volume of 25 µL, included 12.5 µL of Rotor-Gene SYBR Green PCR master mix, 1 µL (400 nM) of each primer, forward and reverse, 8 µL of DNA template, and 2.5 µL of nuclease-free water. PCR was performed using the Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany) with the following settings: hold at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C or 5 s and combined annealing and extension at 60°C for 10 s.

4.3.8 Statistical Analysis.

Cryptosporidium data were analyzed using Tukey HSD test on JMP statistical software. Significant difference of water activity was determined using a one-way analysis of variance (ANOVA) in JMP statistical software. Differences were considered significant when the *P* value was < 0.05.

4.4 Results

4.4.1 ClO_2 Headspace Concentration, Temperature and Relative Humidity.

Gaseous chlorine dioxide treatments were performed in a 35-Liter, light protected polycarbonate vacuum chamber. Three fans were placed inside the chamber

to circulate the gaseous chlorine dioxide which was measures every fifteen minutes. Temperature measurements were taken every five minutes within the chamber, the chamber was 24.28°C ± 0.90°C. Data on relative humidity was collected every five minutes, indicating an average relative humidity of 80.1% and maximum relative humidity of 88.5%.

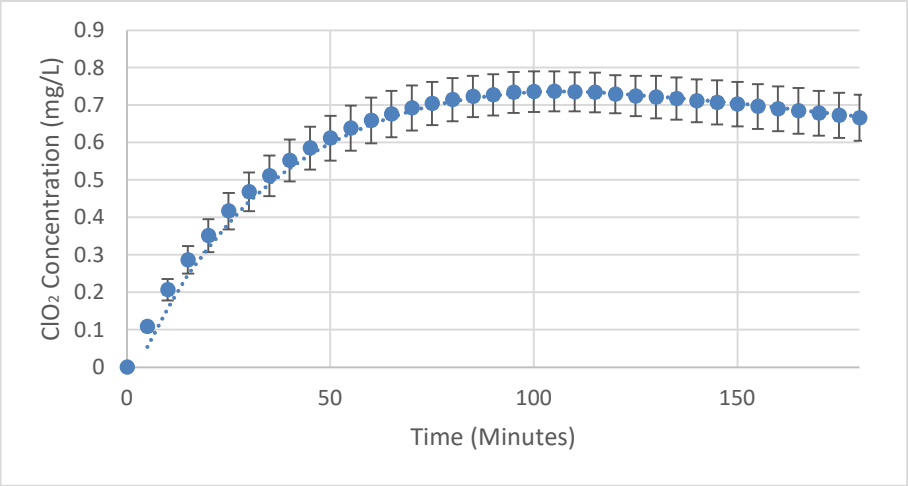

Figure 7. ClO_2 concentration (mg/L) over 180 minutes (3 hours) using an input of 50 grams of each precursor.

4.4.2 Physical Change of Produce

Carrot color change was observed using a colorimeter prior to chlorine dioxide treatment and post-chlorine dioxide treatment. Six carrot samples were measured for overall color change using a colorimeter determines L*, a*, b* values. Using those values delta E^*_{ab}, which is defined as $\sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$ is calculated (Black *et al.,* 2010).

Figure 8. Physical change of carrots after chlorine dioxide treatment of 1 hour and 3 hours. Cross-section of carrot after three-hours of chlorine dioxide treatment.

Exterior carrot color faded significantly after treatment. Looking at the cross-section of the three-hour treated carrot, exterior color change but the interior of the carrot remained unchanged. After the carrot samples were exposed to one hour of gaseous chlorine dioxide treatment, six of each of the colorimeter values were collected and the delta E^*_{ab} calculated was 14.75, a large color change was observed. After three hours of gaseous chlorine dioxide treatment, colorimeter values of carrots were used to calculate the delta E^*_{ab}, 43.46. The overall color change difference between one-hour treatment and three-hour treatment was 15.68.

After gaseous chlorine dioxide treatment, colorimeter data were collected for detection of overall color change. Six of each colorimeter values were taken per time point, to calculate the delta E^*_{ab}. After one hour of gaseous chlorine dioxide treatment, colorimeter values were collected, and the delta E^*_{ab} calculated was 4.01. After three hours of gaseous chlorine dioxide treatment, the delta E^*_{ab} was 1.87, a slight color

change was observed. The overall color change difference of tomatoes between one hour treatment and three-hour treatment was 4.37.

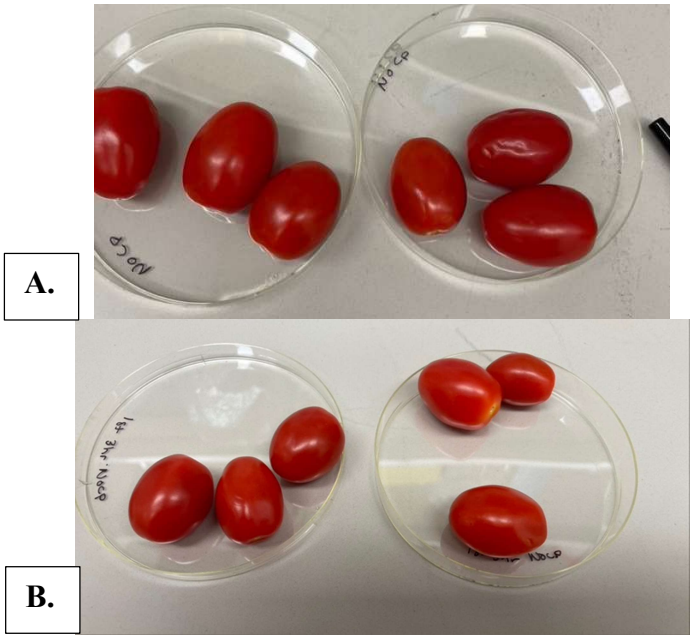

Figure 9. Image A shows grape tomatoes before treatment with gaseous chlorine dioxide. Image B shows grape tomatoes after three hours of treatment with gaseous chlorine dioxide.

Physical changes of grape tomatoes were observed. Image A displays the grape tomatoes prior to chlorine dioxide treatment and image B shows the grape tomatoes after three-hours of chlorine dioxide treatment. There was slight color change and no significant change in moisture content.

4.4.3 Water Activity

Water activity was collected for each produce samples at time point 0, 1 and 3 hours. Water activity from carrot samples at each experimental time point were 0.95±0.014 for 0 hours, 0.96±0.002 one-hour after treatment and 0.95±0.005 after three hours of treatment. Carrots sampled after one hour of gaseous chlorine dioxide treatment for water activity exhibited no significant difference in water activity level ($p=0.0992$). Carrots sampled after three hours of treatment in the chamber showed only a non-significant difference in water activity (0.5632).

Water activity from grape tomato samples at each experimental time point were 0.98±0.003 for 0 hours, 0.98±0.011 one-hour after treatment and 0.98±0.009 after three hours of treatment. Comparing the water activity of grape tomatoes from pre-treatment, zero hours, to after one hour of gaseous chlorine dioxide treatment indicated no difference in water activity ($p=0.3620$). There was also no significant difference ($p=0.2743$) when comparing the water activity of pre-treated grape tomatoes and grape tomatoes after three hours exposure to gaseous chlorine dioxide.

4.4.4 Chlorine Dioxide Residual Test

Once samples are removed from the dissector, control samples were tested for residual chlorine dioxide on the surface of the treated produce. Samples were tested using the Hach Chlorine Dioxide Color Disc Test Kit. Carrot samples tested directly after three-hour treatment showed 0.2 mg/L residual chlorine dioxide and 24hr after treatment carrot samples had zero milligrams per liter residual chlorine dioxide

remaining on produce. There was no residual chlorine dioxide detected on the grape tomato samples, after three hours of gaseous chlorine dioxide treatment, at 0 hours and 24 hours post-treatment.

4.4.5 Reduction of Cryptosporidium parvum

Overall log inactivation of oocysts observed on tomatoes were 4.82 after 1 hour of treatment and at the 3 hours of treatment time *C. parvum* was undetected (Figure 5). For carrots, one hour of gaseous chlorine dioxide treatment resulted in a 3.6-log reduction of *C. parvum.* Carrots that were treated for three hours showed a 5.2-log reduction of *C. parvum.*

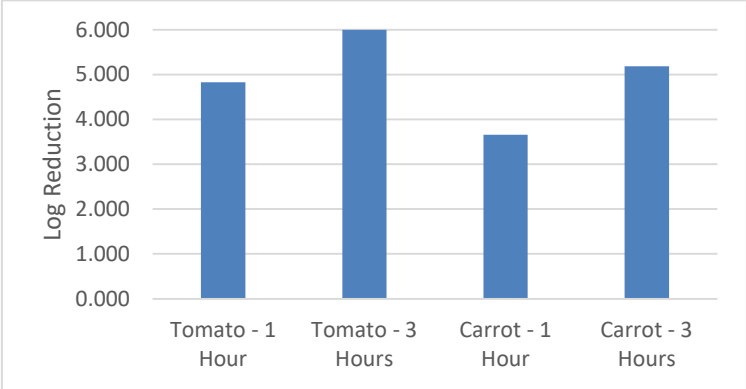

Figure 10. Log reduction of *Cryptosporidium parvum* after treatment with gaseous chlorine dioxide.

4.5 Conclusion

This study accessed the reduction of *Cryptosporidium parvum* in baby carrots and grape tomatoes exposed to gaseous chlorine dioxide. Real-time data was collected including, gaseous chlorine dioxide levels, temperature and relative humidity, congruent with physical change measurements, color and water activity, along with log reduction of *C. parvum* to facilitate in the full understanding of potential for this emerging technology. The results of this study are useful for understanding reduction trends of *C. parvum* throughout treatment with gaseous chlorine dioxides and examining the physical effects of that treatment in terms of food quality.

REFERENCES

Black, E P et al. "Fate of Escherichia coli O157:H7 in ground beef following high-pressure processing and freezing." *Journal of applied microbiology* vol. 108,4 (2010): 1352-60. doi:10.1111/j.1365-2672.2009.04532.x

Chai, H. E., Hwang, C. A., Huang, L., Wu, V. C. H., & Sheen, L. Y. (2020). Feasibility and efficacy of using gaseous chlorine dioxide generated by sodium chlorite-acid reaction for decontamination of foodborne pathogens on produce. Food Control, 108, 106839. https://doi.org/10.1016/j.foodcont.2019.106839

Craighead, Shani et al. "Cold Atmospheric Plasma Jet Inactivates Cryptosporidium parvum Oocysts on Cilantro." *Journal of food protection* vol. 83,5 (2020): 794-800. doi:10.4315/0362-028X.JFP-19-442

Han Y, Selby TL, Schultze KK, Nelson PE, Linton RH. Decontamination of strawberries using batch and continuous chlorine dioxide gas treatments. J Food Prot. 2004 Nov;67(11):2450-5. doi: 10.4315/0362-028x-67.11.2450. PMID: 15553627.

Kingsley, D. H., Pérez-Pérez, R. E., Niemira, B. A., & Fan, X. (2018). Evaluation of gaseous chlorine dioxide for the inactivation of Tulane virus on blueberries. *International journal of food microbiology*, *273*, 28–32. https://doi.org/10.1016/j.ijfoodmicro.2018.01.024

Korich, D. G., Mead, J. R., Madore, M. S., Sinclair, N. A., & Sterling, C. R. (1990). Effects of ozone, chlorine dioxide, chlorine, and monochloramine on Cryptosporidium parvum oocyst viability. *Applied and environmental microbiology*, *56*(5), 1423–1428. https://doi.org/10.1128/AEM.56.5.1423-1428.1990

LeChevallier, Mark W et al. "The potential for health risks from intrusion of contaminants into the distribution system from pressure transients." *Journal of water and health* vol. 1,1 (2003): 3-14.

Lee, S.Y., Costello, M., & Kang, D.H. (2004). Efficacy of Chlorine Dioxide Gas as a Sanitizer of Lettuce Leaves. Journal of Food Protection, 67(7), 1371–1376. https://doi.org/10.4315/0362-028X-67.7.1371

Ortega, Y. R., A. Mann, M.P. Torres, and V. Cama (2008). Efficacy of Gaseous Chlorine Dioxide as a Sanitizer against Cryptosporidium parvum, Cyclospora cayetanensis, and Encephalitozoon intestinalis on Produce. J. Food Protect., 71(12), 2410–2414. doi:10.4315/0362-028X-71.12.2410

Rane, B., Bridges, D. F., & Wu, V. (2020). Gaseous antimicrobial treatments to control foodborne pathogens on almond kernels and whole black peppercorns. *Food microbiology*, *92*, 103576. https://doi.org/10.1016/j.fm.2020.103576

Sy, K. V., McWatters, K. H., & Beuchat, L. R. (2005). Efficacy of gaseous chlorine dioxide as a sanitizer for killing Salmonella, yeasts, and molds on blueberries, strawberries, and raspberries. Journal of Food Protection, 68(6), 1165–1175. https://doi.org/10.4315/0362-028X-68.6.1165

Appendix A

CONFIRMATION OF THE PRESENCE OF CRYPTOSPORIDIUM PARVUM IN PRESUMPTIVE POSITIVE WATER SAMPLES USING NESTED PCR AND RESTRICTION FRAGMNT LENGTH POLYMORPHISM ANALYSIS

A.1 Abstract

Cryptosporidium parvum and hominis are *Cryptosporidium* species that infect humans which could be life threatening for young children and immunocompromised persons. In 2016, cryptosporidiosis is the leading cause of diarrheal morbidity and mortality in children younger than 5 years old and acute infection caused more than 48,000 deaths. A total of seventy-two water samples were collected regularly from various sites in Delaware and Maryland over the course of 17 months (June 2017-October 2018). DNA Primer Sequences for *Cryptosporidium* Genus-specific PCR Amplification (18S rRNA) used, followed by further testing using *Vsp*I and *Dra*II restriction fragment length polymorphism enzyme for speciation confirmation. The nested PCR amplicons were digested with one unit of *Vsp*I for distinction between *C. parvum* and *C. hominis*. For distinction of *C. parvum, C. baileyi* and *C. serpenti*, the nested PCR amplicon were digested with one unit *Dra*II. Twenty-four out of seventy-two water samples were confirmed to contain *Cryptosporidium parvum*. Through further testing using restriction fragment length polymorphism analysis, one water sample was confirmed to harbor *C. cayetanensis* or *E. neischulzi,* which have genetic similarities to *Cryptosporidium* species. A single sample was identified to be either *C.*

baileyi or *C. serpenti,* which do not infect humans. Knowledge of parasitic organisms in potential irrigation water sources are essential for correct implementation of water testing and water treatment.

Highlights

- 33.3% of water collected in the mid-Atlantic region contained *C. parvum*.
- RFLP analysis identified a non-*Cryptosporidium* sample as either *C. cayetanensis* or *E. neischulzi*
- One sample, out of 72, was identified as either *C. baileyi* or *C. serpenti.*

A.2 Introduction

Cryptosporidiosis is a disease infects humans causing infection by *Cryptosporidium parvum or Cryptosporidium hominis*. Symptoms include nausea, vomiting, watery diarrhea, dehydration, weight loss and stomach pain. Cryptosporidiosis symptoms can be life threatening for immunocompromised individuals. In 2016, *Cryptosporidium* infection was the leading cause of diarrheal morbidity and mortality in children younger than 5 years old and acute infection caused more than 48,000 deaths (Khalil *et al.,* 2018).

The objective of this study was to collect data on *Cryptosporidium* species from the Mid-Atlantic Region for environmental monitoring and risk assessment. The *Cryptosporidium* species, *C. parvum* and *C. hominis,* most commonly infects humans. Less common *Cryptosporidium* species, *C. baileyi* and *C. serpenti,* infect immunocompromised individuals. Prevalence of *Cryptosporidium* species varies by

water type including, highest prevalence in wastewater (46.9%), as well as, 45.3% in surface water and 31.6% in raw water (Daraei et al., 2021).

Traditional detection techniques are unable to specify different *Cryptosporidium* species. Speciation of *Cryptosporidium* is essential because not all species infect humans. Recent studies have classified *C. parvum* into genotype I, found in humans, and genotype II, infect both humans and animals.

The PCR method was designed as a nested primer set for *C. parvum* specificity and sensitivity of detection with limited number of oocysts as described in Sturbaum *et al.,* 2001. Primary amplification through external primer set amplifies 844- and 840-basepair fragments from genotype I and genotype II, respective. Nested amplification amplifies 593- and 590-basepairs, respective. Differentiation between *C. parvum* genotype 1 and genotype 2 occurs by restriction fragment length polymorphism using the restriction enzyme *Vsp*I. Resulting in 503- and 90-basepair fragments for genotype 1 and genotype 2 results in no fragments because there is no restriction cut sites. Differentiation of *C. parvum* from *C. baileyi* and *C. serpentis* through restriction fragment length polymorphism using the restriction enzyme *Dra*II. Presence if *C. baileyi* results in 295- and 284-basepair fragments and presence of *C. serpentis* results in 298- and 284-basepair fragments.

A.3 Methods

Samples were previously collected from the CONSERVE project which were processed by filtration using a modified EPA Method 1623: *Cryptosporidium* and

Giardia in Water by Filtration. The modification to the EPA methods included DNA extraction and quantitative polymerase chain reaction (qPCR) in place of immunomagnetic separation (IMS) and fluorescent antibody assay (FA). Water samples were collected regularly from various sites in Delaware and Maryland over the course of 17 months (June 2017-October 2018). Seventy-two water samples were collected in total. DNA was extracted from water samples using the DNeasy PowerWater Kit (Qiagen, Hilden, Germany). The presumptive presence of *C. parvum* was determined using quantitative PCR with QuantiNova Probe PCR Kit (Qiagen, Hilden, Germany). PCR detection of *C. parvum* included use of forward primer (CPHSPT2F, 5' TCC TCT GCC GTA CAG GAT CTC TTA 3') and reverse primer (CPHSPT2R, 5' TGC TCT TAC CAG TAC TCT TAT CA 3') (LeChevallier et al., 2003). The PCR reaction volume of 25 µL, included 12.5 µL of Rotor-Gene SYBR Green PCR master mix, 1 µL (400 nM) of each primer, forward and reverse, 8 µL of DNA template, and 2.5 µL of nuclease-free water. PCR was performed using the Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany) with the following settings: hold at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C or 5 s and combined annealing and extension at 60°C for 10 s. A standard curve was generated in duplicate for each qPCR run.

Table 3: DNA Primer Sequences for *Cryptosporidium* Genus-specific PCR Amplification (18S rRNA)

Primer Code	Primer Specificity	Primer Sequence (5'-3')	Amplicon Size (bp)	Application
ExCry1 (Forward)	*Cryptosporidium* spp.	GCCAGTAGTCATATGCTTGTCTC	840-844	Primary Amplification
ExCry2 (Reverse)	*Cryptosporidium* spp.	ACTGTTAAATAGAAATGCCCCC	840-844	
NesCry3 (Forward)	*Cryptosporidium* spp.	GCGAAAAAACTCGACTTTATGGAAGGG	590-593	Nested Amplification
NesCry4 (Reverse)	*Cryptosporidium* spp.	GGAGTATTCAAGGCATATGCCTGC	590-593	

Restriction fragment length polymorphism was used to distinguish *Cryptosporidium* species. The nested PCR amplicons were digested with one unit of *Vsp*I for distinction between *C. parvum* and *C. hominis*. For distinction of *C. parvum, C. baileyi* and *C. serpenti*, the nested PCR amplicon were digested with one unit *Dra*II. Samples were then analyzed by gel electrophoresis using a 3% NuSieve gel prepared with 0.5x TBE and 0.2% ethidium bromide. The restriction fragment length polymorphism patterns were visualized on agarose gel by using a UV transilluminator.

Table 4: PCR and Restriction Fragment Length Polymorphism Size for Distinguishing Cryptosporidium species.

Organism	PCR Amplicon		RFLP Digestion Products (bp)	
	Primary (bp)	Nested (bp)	*Vsp*I	*Dra*II
C. hominis (formerly C. parvum, genotype I)	844	593	503 and 90	-
C. parvum (formerly C. parvum, genotype II)	840	590	-	-
C. baileyi	831	579	-	295 and 284†
C. serpentis	836	583	-	298 and 284†
C. muiris	-	-	-	-
C. wrairii	-	-	-	-

†Indistinguishable within an agarose gel

A.4 Results

Twenty-six presumptive positive water samples were further tested to confirm presence of *Cryptosporidium parvum.* All 26 samples tested positive for the primary amplification. Twenty-five out of the original 26 samples tested positive for the nested amplification. Sample (#43) resulted in positive primary amplification and negative nested amplification could indicate presence of either *Cyclospora cayetanensis* or *Eimeria neischulzi* (Sturbaum *et al.,* 2001).

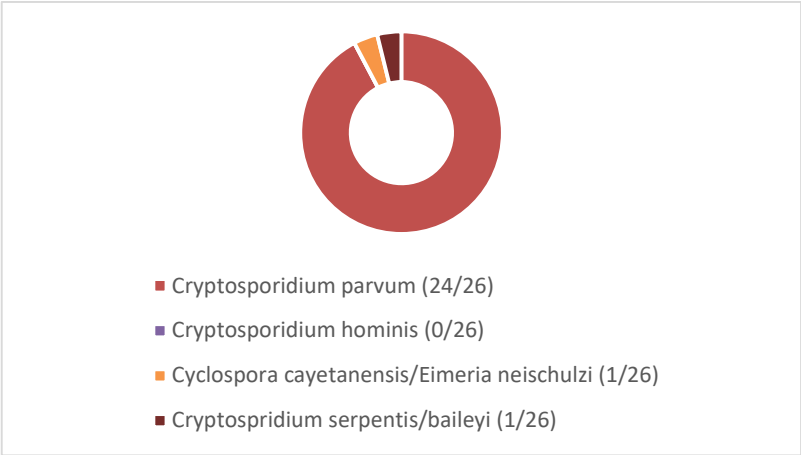

Figure 11. Results from species-specific nested PCR-restriction fragment length polymorphism detected 24 of the 26 presumptive positive water samples contained *C. parvum.* During further testing using RFLP digestion with *Dra*II, one sample was identified as *Cryptosporidium serpentis* or *baileyi.* One sample was identified as non-*Cryptosporidium* species, could indicate the presence of either *Cyclospora cayetanensis* or *Eimeria neischulzi*.

There were zero samples that amplified base pair fragments from the RFLP digestion with *Vsp*I, therefore, none of water samples that were collected contain *Cryptosporidium hominis,* previously known as *C. parvum* genotype I. Twenty-four of out the seventy-two water samples collected resulted in positive results for the primary amplification, positive results for the nested amplification and negative results for both RFLP digestions. These results indicate presence of *C. parvum,* previously known as *C. parvum* genotype II.

One sample had positive results for the RFLP digestion with *Dra*II. That sample (#54) resulted in positive results for primary amplification, nested amplification and RFLP digestion with *Dra*II but negative results for RFLP digestion

with *Vsp*I. Those results indicate presence of either *C. serpentis* or *C. baileyi* (Sturbaum *et al.,* 2001).

A.5 Conclusion

Environmental sampling of agricultural water used for irrigation is important protecting public health and reinforces the need for pre-harvest pathogen remediation technologies. With increased need for alternative water sources for irrigation purposes, characterizing the protozoan population of alternative water sources are paramount for crop production and preventing illness. Approximately 33.3% of the water samples collected in the mid-Atlantic region (n=72) tested positive for *Cryptosporidium parvum*. Knowledge of parasitic organisms in potential irrigation water sources are essential for correct implementation of water testing and water treatment.

REFERENCES

Daraei, Hasti et al. “Prevalence of Cryptosporidium spp. in water: a global systematic review and meta-analysis.” Environmental science and pollution research international vol. 28,8 (2021): 9498-9507. doi:10.1007/s11356-020-11261-6

Khalil, Ibrahim A et al. “Morbidity, mortality, and long-term consequences associated with diarrhoea from Cryptosporidium infection in children younger than 5 years: a meta-analyses study.” *The Lancet. Global health* vol. 6,7 (2018): e758-e768. doi:10.1016/S2214-109X(18)30283-3

LeChevallier, Mark W et al. “The potential for health risks from intrusion of contaminants into the distribution system from pressure transients.” *Journal of water and health* vol. 1,1 (2003): 3-14.

Sturbaum, G D et al. “Species-specific, nested PCR-restriction fragment length polymorphism detection of single Cryptosporidium parvum oocysts.” Applied and environmental microbiology vol. 67,6 (2001): 2665-8. doi:10.1128/AEM.67.6.2665-2668.2001