



## **CPS 2019 RFP FINAL PROJECT REPORT**

### **Project Title**

Analysis of the presence of *Cyclospora* in waters of the Mid-Atlantic States and evaluation of removal and inactivation by filtration

### **Project Period**

January 1, 2020 – December 31, 2020 (extended to May 2021)

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### **Objectives**

1. *Confirmation of the presence of Cyclospora cayetanensis in presumptive positive environmental water samples using the Sanger (dideoxy) sequencing method.*
2. *Evaluation of zero-valent iron (ZVI) and sand filtration in the removal and inactivation of C. cayetanensis surrogates in artificial agricultural water.*

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## FINAL REPORT

### 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 and does not yield simple answers. Given the difficulties with detection, and the potential for contamination based on epidemiological studies, intervention strategies for water are valuable. Filtration using a laboratory filtration unit containing sand and zero-valent iron (ZVI) can successfully remove oocysts from contaminated pond water. In this project, filtration using the laboratory-scale ZVI-sand column achieved a 4.3 log reduction of *Cryptosporidium parvum* oocysts compared to a 1.8 log reduction using a sand-only filter. Similarly, a ZVI-sand filter resulted in a 6-log removal of *Eimeria tenella* oocysts compared to an average 2.3 log removal by a sand filter. This project provides evidence of the need for continued study of *Cyclospora* detection and removal methodologies.

### Background

*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. 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, namely *C. cayetanensis* (Ortega and Sanchez, 2010; Lainson, 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 relationships to organisms of the *Eimeria* genus as well as a strong relation to *Toxoplasma* (Li et al., 2017; Cinar et al., 2015; Zhao et al., 2013).

This project has two main objectives that address 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 is 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 included 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. This project included further analysis of these samples by amplicon (PCR product) sequencing and subsequent analysis. The second objective includes testing the effectiveness of ZVI filtration in the removal and inactivation of parasitic pathogens, to improve pre-harvest food safety. This work will also 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 reducing parasitic foodborne illness. The proposed ZVI technology may offer advantages to small and mid-sized growers, including adaptation to existing filtration systems, feasibility, effective filtration and broad neutralization of numerous biological and chemical hazards, with minimal environmental impacts.

## Research Methods and Results

### Methods – Objective 1

#### Sample collection and detection

(While this part of the project was not funded by CPS, it is critical to the project.)

Water samples were initially collected and studied for the presence of several protozoan parasites, including *Cryptosporidium*, *Giardia intestinalis*, *Toxoplasma gondii*, and *Cyclospora cayetanensis*. Seventy-two water samples were collected from six sites in June 2017 through September 2018. Samples were collected biweekly during fruit and vegetable growing seasons (June to October 2017 and May to September 2018) and then samples were collected once during the non-growing season time periods (November 2017 to April 2018, and October 2018). Water types included surface (pond and tidal river water) and reclaimed (vegetable processing water and treated wastewater). Physicochemical data were measured in triplicate with EXO2 or ProDSS multiparameter water quality sonde/meter (data not included in this report, but available upon request). Samples were transported to the laboratory for immediate filtration or stored at 4°C for up to 24 hours. Up to 20 L of each water sample were agitated and then filtered, concentrated, and pelleted in accordance with the EPA 1623.1 method. DNA was extracted from pellets using DNeasy® PowerWater® kits (Qiagen, Hilden, Germany), and performed in accordance with recommendations and instructions from the manufacturer. 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. Real-time PCR was performed using the QuantiNova™ Probe PCR kit (Qiagen) according to manufacturer's instructions. For all water samples, if one replicate of the unknown sample was positive, the sample was considered positive. 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 and DeSilva, 2016).

#### Presumptive positive sample analysis, sequencing, and sequence 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., 2003) (**Table 1**).

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 ABI Prism 3130XL Genetic Analyzer (Applied Biosystems). Data were analyzed by assistance of a computational biologist, Dr. Shawn Polson, Director of the University of Delaware Bioinformatics Center. Chromatograms were imported into Geneious (v 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).

## Results – Objective 1

Nested PCR amplification was performed on all presumptive positive water samples. Primers were tested using DNA from *C. cayetanensis*, *Eimeria acervulina*, *E. maxima*, and *E. tenella* as positive controls. Nuclease-free water, and DNA from *Cryptosporidium parvum* 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. Based on initial analysis, one sample showed 99.9999% similarity to *C. cayetanensis* based on the sequence that is in GenBank; however, after further review this sample appears to include multiple sequences, including an 80 out of 81 base pair match to *C. cayetanensis*. Due to the remaining ambiguous base pairs in this sample, it is still under review. 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 calls at most positions, but a significant number of positions showed a mixed signal. 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). These issues continue to be assessed.

Several phylogenetic trees were created to better understand the similarities across these samples and including the top 10 to 25 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 that are all the descendants of a common ancestor. The phylogenetic tree included in **Figure 1** shows the evolutionary distance of these organisms. There is greatest similarity in the *Cyclospora* gene fragment (indicated as tCL) and 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 finding in this dataset that this apicomplexan group of protozoa is difficult to speciate is supported by the literature. 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. Phylogenetic analysis will continue on these

samples with the assistance and guidance of Dr. Shawn Polson, and final results will be included in a future publication.

## Methods – Objective 2

### Protozoa preparation

*Cryptosporidium parvum* oocysts were purchased from Waterborne Inc. (New Orleans, LA) and *Eimeria tenella* oocysts were a gift from Dr. Mark Jenkins at the USDA-ARS Animal Parasitic Diseases Laboratory (Beltsville, MD). All protozoa were stored at 4°C and used within 3 months of receiving.

### Sand and sand:ZVI column construction and utilization

Sand columns, which served as a control, and sand:ZVI columns, referred to as test columns, were constructed in pairs as previously described (Shearer and Kniel, 2018). A steel mesh disk (60 mesh; 60 openings per in<sup>2</sup> mesh, wire diameter 0.0075 in.) was placed at the bottom of each acrylic column (3.8 inside diameter by 10 cm). Columns were wet packed to the top with sterile deionized water and sand alone (for the control columns) or a 1:1 (w/w) mixture of sand and ZVI (particle size 0.005 to 0.125 in.). Columns were sealed with tubing affixed at the center of the top and bottom.

A 3-L volume of sterile water was passed over the columns before the start of the filtration trials. Each organism was analyzed in triplicate, meaning that 3 trials were performed for each of the two protozoa studied. 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 ( $1 \times 10^6$ ) in autoclaved pond water; 3) three individual pulses, of 45 mL each, of autoclaved pond water (3 x 45 mL). Liquid was pumped through the columns at a rate of 1 mL/min by using a Tris Peristaltic pump. Throughout the filtration study 5-mL fractions of eluate were collected in sterile glass tubes with a Foxy Jr. fraction collector. Fractions were pooled (3 tubes in each subsample, 15 ml) and stored at 4°C. Studies were performed in triplicate, control groups include a negative control, and a positive control. Removal and inactivation of oocysts were assessed using qPCR.

### Identification of *Cryptosporidium parvum* oocysts and infectious *Cryptosporidium*

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. The reduction in infectivity of *C. parvum* oocysts was quantified by an integrated cell culture and qPCR assay. *Cryptosporidium* oocysts were bleached, and triple rinsed with Hanks balanced salt solution (HBSS) prior to filtration. Untreated oocysts and nuclease-free water were used as positive and negative controls. Human cell culture was infected with oocysts as described previously (Craighead et al., 2020). In brief, filtrate samples were pooled as described above, and then were applied to HCT-8 cell confluent monolayers in a 6-well cell culture dish, incubated for 2 h, and then cells were triple rinsed with HBSS. Infected cells were incubated at 37°C with 5% CO<sub>2</sub> for 48 h. DNA extraction was completed using the Zymo Research Quick-DNA Extraction kit. Then qPCR was performed with the Qiagen SYBR Green PCR kit and Rotor-Gene Q cycler (Qiagen). In addition, each sample was run in duplicate in qPCR. A standard curve was generated using control oocysts, which was then imported and adjusted for each qPCR run. 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 uL, included 12.5 uL of Rotor-Gene SYBR Green PCR master mix, 1 uL (400 nM) of each primer, forward and reverse, 8 uL of DNA template, and 2.5 uL of nuclease-free water. PCR was performed using the Rotor-Gene Q

thermocycler (Qiagen) with the following settings: hold at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 5 s and combined annealing and extension at 60°C for 10 s.

### **Identification of *Eimeria tenella* oocysts**

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. 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, 0.5 µM), 0.4 µM stock of ET-R (final concentration, 0.5 µM), and 7.2 µl of 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 30 s at 95°C, 20 s at 62°C and 20 s at 72°C. A melting curve program involved heating from 60 to 95°C at a rate of 0.1 °C/s and was performed to create the dissociation curve.

### **Statistical analysis**

Data were analyzed using a Matched Pairs test in JMP software. Differences were considered significant when the *P* value is < 0.05.

## **Results – Objective 2**

### **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. After filtration through either sand or a 1:1 sand and ZVI mixture, detection levels of *C. parvum* were obtained using 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 99.9% reduction of *C. parvum* oocysts as determined by detection of genomic copies over the filtration study. Filtration using the sand:ZVI column achieved a 4.30 log reduction of *C. parvum* compared to 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. This is shown in **Figure 2**.

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. These analyses show 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.

### **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 filtrate from either sand or 1:1 sand and ZVI columns. Data are the average of three trials. Filtration with a sand and ZVI mix column resulted in no detectable *E. tenella*, as determined by zero genomic copies per reaction in all samples tested. Filtration using sand-only columns resulted in a 99.5% reduction of *E. tenella*, as determined by analysis of genomic copies across all samples. From total analysis, sand:ZVI filtration achieved a 6 log reduction, whereas sand-only filtration reduced *E. tenella* by 2.3 log. This is shown in **Figure 3**.

## **Outcomes and Accomplishments**

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 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 of *Cyclospora cayetanensis*. An accomplishment of this project is providing evidence that sand filtration provides efficient removal of small and large protozoa and inclusion of ZVI with sand can increase removal efficiencies of both *Cryptosporidium* and *Eimeria*.

### **Summary of Findings and Recommendations**

Research with *C. cayetanensis* is severely limited by the lack of resources and inability to obtain *C. cayetanensis* oocysts. Throughout history, under such conditions scientists have garnered vast amounts of data through the use of surrogate microorganisms. In this study, two protozoan surrogates were used. *Cryptosporidium* is a robust pathogen resistant to chemical disinfectants but smaller in size compared to *Cyclospora*. *Eimeria tenella* is a bit larger than *Cyclospora* but more similar in morphology to *Cyclospora*. Together these two pathogens provide useful information.

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 biologies 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.

Sand biofilters 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. The project findings suggest that zero-valent iron can enhance the efficacy of filtration for removing protozoa of varying sizes (5 to 22 microns). Future research should address the inactivation of protozoan oocysts, including feasibility and mechanistic studies.

## **APPENDICES**

### **Publications and Presentations**

#### Presentations:

A. Kelly, Confirmation of the presence of *Cyclospora cayetanensis* in presumptive positive environmental water samples, Seminar presentation to the Department of Animal and Food Sciences, 3/29/2021.

A. Kelly, S. Craighead and K.E. Kniel, Confirmation of the Presence of *C. cayetanensis* and *C. parvum* from Environmental Water Samples, International Association for Food Protection Annual Meeting, Phoenix, Arizona, July 2021, P2-173.

### **Budget Summary**

The project was severely impacted by the COVID-19 pandemic, which resulted in the closure of the University of Delaware and suspension of the majority of laboratory work from March to September 2020. This study continued to be affected by the COVID-19 pandemic after that period due to the protective safety rules in place at the University allowing low numbers of laboratory personnel in at one time. The project was additionally impacted by a move to a new laboratory, that had to be rescheduled for January 2021 from its previously scheduled time due to the pandemic and resulted in an additional 6 weeks of laboratory closure. The delays impacted spending on personnel and some supplies for some studies. The need to include funds spent on bioinformatics support became obvious following initial analysis of the sequencing data. The scope of the project did not change, and we will continue data analysis through to the publication of this work. A total of \$49,170 in research funds was awarded to this project. The majority of funds were spent within the past couple of months due to constraints in the laboratory associated with the physical move and COVID-19 restrictions.

We would like to thank CPS for the no-cost extensions granted in this project associated with loss of productive time due to the pandemic. We regret that the delays impacted this project, as all were beyond the control of the research team.

**Table 1 and Figures 1–3** (see below)

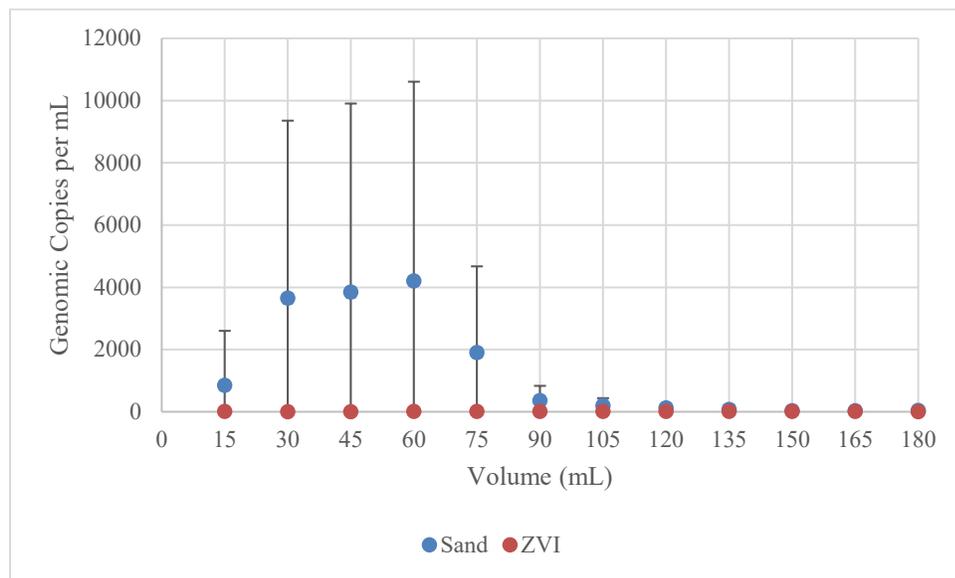
**Table 1:** DNA Primer Sequences for *Cyclospora*-specific PCR Amplification (18S rRNA gene). These are used in the nested PCR analysis, adapted from Orlandi et al., 2003.

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

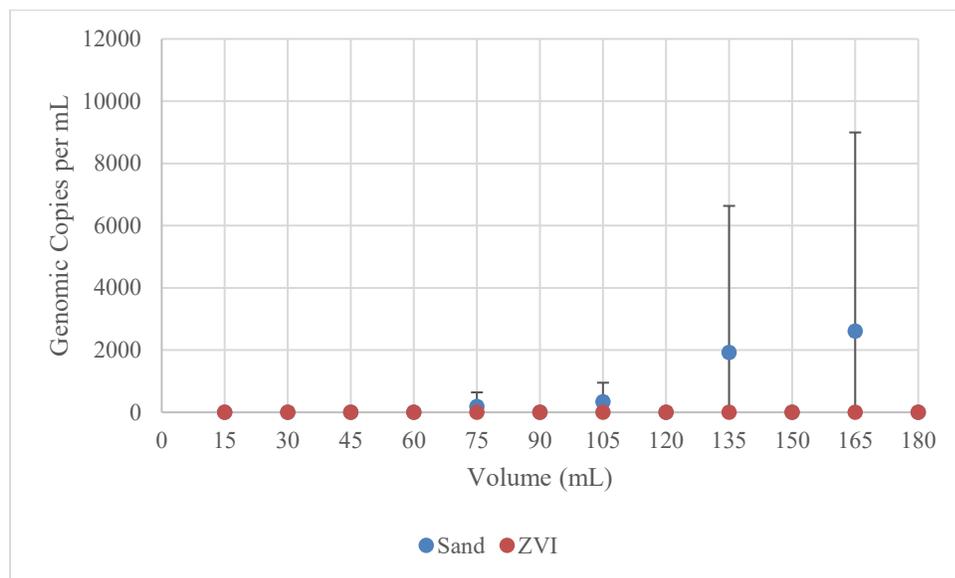
**Figure 1.** Phylogenetic tree representing NCBI blast results showing genetic similarities to presumptive positive environmental water samples identified as *C. cayetanensis* through nested PCR (numerical entries) with *Toxoplasma gondii* included as an out-group. The sample tCL is the control gene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



**Figure 2.** 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.



**Figure 3.** 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.



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