



Published in final edited form as:

J Microbiol Methods. 2025 September ; 236: 107195. doi:10.1016/j.mimet.2025.107195.

Evaluation of nucleic acid extraction methods for recovery of *Cyclospora cayetanensis*, *Salmonella enterica*, and murine norovirus from water and sludge

Amy Kahler^{a,1}, Jessica Hofstetter^{a,b,c,1}, Camila Rodrigues^b, Mia Mattioli^{a,*}

^aWaterborne Disease Prevention Branch, Division of Foodborne, Waterborne, and Environmental Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Road. MS H23-9, Atlanta 30329, GA, United States of America

^bDepartment of Agriculture, Auburn University, 101 Funchess Hall, Auburn, AL 36849, United States of America

^cChenega Systems and Solutions, 609 Independence Parkway, Suite 210, Chesapeake, VA 23320, United States of America

Abstract

The coccidian parasite *Cyclospora cayetanensis* is the causative agent for foodborne outbreaks of cyclosporiasis and multiple fresh produce recalls annually. In recent years, this organism has been reported in the water near produce growing operations during outbreak investigations, prompting a call for more research on its environmental prevalence in the United States. Currently, there is a lack of performance data available on methods for conducting this research, including the performance of DNA extraction methods for molecular testing. Extraction methods for environmental samples must be efficient due to the often-limited amount of target nucleic acid and the potential for molecular inhibitors present in an environmental sample. This study assessed the performance of *C. cayetanensis* nucleic acid extraction seeded into surface water, produce wash water, and tap water by two methods designed for use with environmental samples: the PowerViral and UNEX methods. The PowerSoil extraction method (2 g) was assessed for *C. cayetanensis* extraction from seeded sewage sludge – an environmental sample type used to evaluate parasite carriage within communities. Extraction performance of the PowerViral and UNEX methods were also assessed for the detection of the foodborne bacterial pathogen *Salmonella* and a surrogate for foodborne viruses, murine norovirus (MNV) seeded into surface water, produce wash water, and tap water. The PowerViral method resulted in consistent detection (83–100 %) of *C. cayetanensis*,

This is an open access article under the CC BY-NC license (<http://creativecommons.org/licenses/by-nc/4.0/>).

*Corresponding author. kuk9@cdc.gov (M. Mattioli).

¹Co-first authors contributed equally to this manuscript.

CRediT authorship contribution statement

Amy Kahler: Writing – original draft, Methodology, Investigation, Conceptualization. **Jessica Hofstetter:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Camila Rodrigues:** Writing – review & editing. **Mia Mattioli:** Writing – review & editing, Supervision, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

S. enterica, and MNV across all water types. Detection rates for the UNEX method ranged from 56 to 100 % prevalence for tap water and wash water, but there were no detections for any microbe from surface water. The PowerSoil method resulted in poor recovery of *C. cayetanensis* from sludge (≤ 1 % recovery), while both the PowerViral and UNEX methods effectively recovered *C. cayetanensis* from sludge (4–36 % recovery).

Keywords

Cyclospora cayetanensis; *Salmonella enterica*; Murine norovirus; DNA extraction; RNA extraction; Molecular methods

1. Introduction

Cyclospora cayetanensis is a coccidian parasite that causes the foodborne illness cyclosporiasis. *C. cayetanensis* is excreted as an environmentally resistant oocyst from the infected human host (Centers for Disease Control and Prevention, 2021; Quintero-Betancourt et al., 2002). United States outbreaks of cyclosporiasis were typically linked to fresh produce grown in endemic countries outside of the U.S. until outbreaks in 2018 and 2020 were associated with domestically grown produce (Abanyie et al., 2015; Hadjilouka and Tsaltas, 2020; U.S Food and Drug Administration, 2020, 2021). While fresh produce is often identified as the source of cyclosporiasis outbreaks, oocysts of *C. cayetanensis* require, on average, seven to fourteen days to sporulate into their infectious state after shedding from the host (Smith et al., 1997). This makes food contamination from harvest workers or person-to-person transmission unlikely, but instead agricultural waters could serve as an effective vehicle for contaminating growing produce and allow for sporulation to occur prior to consumption. To effectively evaluate U.S. produce irrigation waters for *C. cayetanensis*, the performance of testing methods for *C. cayetanensis* in environmental samples must be understood.

The most sensitive methods available for the detection of *C. cayetanensis* in environmental samples employ real-time, quantitative polymerase chain reaction (qPCR); therefore, efficient nucleic acid extraction and purification is an important first step in detection. Extraction is particularly important for effective *C. cayetanensis* detection because rupturing the chemically resistant oocyst wall is necessary for nucleic acid release (Erickson and Ortega, 2006; Qvarnstrom et al., 2018a; Shields et al., 2013). Nucleic acid extraction method performance can greatly affect the subsequent interpretation of *C. cayetanensis* qPCR data and different commercial kits have been shown to yield varying DNA output quantities, qualities, and subsequent target amplification efficiencies (Almeria et al., 2018; Assurian et al., 2020b; Iker et al., 2013; O'Brien et al., 2021; Qvarnstrom et al., 2018a; Shields et al., 2013; Zielińska et al., 2017). Factors to consider when choosing an extraction method include: sample matrix, sample volume to be extracted, target organism type (e.g., parasite, bacteria, and/or virus), target nucleic acid type (e.g., RNA and/or DNA), and predicted target concentration in tested samples (Thatcher, 2015). Moreover, environmental water concentrates contain high amounts of naturally occurring substances that could act to inhibit PCR. These include humic substances, which are the product of microbial breakdown of

plant material and are co-concentrated with the molecular targets (Abbaszadegan et al., 1993; Gentry-Shields et al., 2013). Humic substances interfere with PCR by chelating the ions needed for the polymerase and have been shown to interfere with the molecular reaction (Tsai and Olson, 1992). For this reason, an inhibitor removal step is beneficial to an environmental nucleic acid extraction technique.

Previous research assessing the performance of extraction methods for *C. cayetanensis* detection have largely focused on extraction from food or clinical samples (Qvarnstrom et al., 2018a; Shields et al., 2013; Temesgen et al., 2020). However, the utility of these methods for environmental water and sewage sludge samples is unknown, as previous methodological studies using environmental samples have emphasized the performance of sample concentration, oocyst isolation or molecular detection methods, neglecting the assessment of DNA extraction efficacy (Borchardt et al., 2009; Kahler et al., 2021; Shields and Olson, 2003; Totton et al., 2021).

Our study assessed the efficacy of two extraction methods for detecting *C. cayetanensis* in three water types with varying levels of organic matter and inhibitors – surface, produce wash, and tap – as well as sewage sludge, which was previously tested to evaluate parasite carriage within communities (Kahler et al., 2024). To evaluate the performance for co-extraction of other food safety related microbial targets, all water samples were also seeded with the gram-negative bacteria, *Salmonella enterica*, and an RNA, enteric virus surrogate, murine norovirus-1 (MNV). Two extraction methods were evaluated for water samples: the Qiagen AllPrep® PowerViral® DNA/RNA kit (Qiagen, Hilden, Germany) and the Universal Nucleic Acid Extraction (UNEX) method (Hill et al., 2015). Three extraction methods were evaluated for sewage sludge samples: the two evaluated for water samples and the Qiagen RNeasy PowerSoil Total RNA kit with the RNeasy PowerSoil DNA Elution kit. These three extraction methods were selected due to their ability to co-extract DNA and RNA, incorporate bead beating steps to facilitate cell lysis, and utilize inhibitor removal mechanisms. The three extraction methods evaluated were designed for different input volumes, which can impact the sensitivity of pathogen detection. The PowerViral and UNEX methods require smaller input volumes, 200 µL and up to 750 µL, respectively, while the PowerSoil method accepts larger amounts (2 g). Two method variations were evaluated for the PowerViral and UNEX methods. Two input volumes were assessed to evaluate whether increasing sample volume negatively impacted extraction efficiency. Two types of beads for bead beating were assessed to evaluate the impact of bead composition on extraction efficiency.

2. Materials and methods

2.1. Environmental sample collection and concentration

Surface and tap water samples were collected from September to November 2020 from streams, ponds, and buildings in DeKalb County, Georgia, U.S. Produce wash water and municipal sewage sludge samples were collected between January 2020 through December 2021 in Tift County, GA. Sample processing and nucleic acid extraction were conducted at the Centers for Disease Control and Prevention (CDC) in Atlanta, GA.

Tap water (100L) was collected at two different time points (two samples) directly from a municipal-fed tap by dead-end ultrafiltration (DEUF) following previously described methods (Kahler et al., 2015). Prior to ultrafiltration, tap water samples were treated with 0.01 % sodium polyphosphate (NaPP) (Sigma-Aldrich No. 305553, St. Louis, MO) to neutralize free chlorine residual. Surface water samples (50–75 L) were collected on different days from a small tributary to the South Fork Peachtree Creek ($N = 3$) and from Murphey Candler Lake in Atlanta, GA ($N = 1$). Surface waters were ultrafiltered similar to tap water, and all ultrafilters were backflushed as previously described (Kahler et al., 2021). Surface and tap water sample backflush was then further concentrated by centrifugation at 4000 $\times g$ for 15 min. All but 10 mL of the supernatant was removed, which was then used to resuspend the pelleted sample. The resulting concentrates were stored at -20°C until microbe seeding and nucleic acid extraction.

Spent produce wash water was collected at two separate time points (two samples) by pumping from the produce dump tank into a 20-L cubitainer at a packing house facility. Water was concentrated by continuous flow centrifugation (CFC) as previously described (Kahler et al., 2021). CFC eluates were further concentrated by centrifugation at 4000 $\times g$ for 15 min. The resulting concentrates (15 mL) were stored at -20°C until microbe seeding and nucleic acid extraction.

Municipal sludge samples (1 L) were collected from two locations in the wastewater treatment plant processing system at three separate time points for each sampling location. The two locations include the thickener recovery system (REC) used to generate sludge for land application ($N = 3$) and the return activated sludge (RAS) from the aeration basin ($N = 3$). Samples were frozen at -20°C upon arrival at CDC until further processing (3–19 months). Thawed sludge samples were heat inactivated in a 60°C water bath for one hour. After cooling, approximately 500 mL was concentrated by centrifugation at 4000 $\times g$ for 15 min. The supernatant was removed, and sludge concentrates were stored at -20°C until microbe seeding and nucleic acid extraction.

2.2. Microbe preparation and sample seeding

Flow-sorted *C. cayetanensis* oocysts were purified from human stool pooled specimens collected in Indonesia, and oocysts were quantified into aliquots of one million oocysts following previously published methods (Qvarnstrom et al., 2018b). The sporulation status of the oocysts was observed on a Zeiss Primostar microscope at $40\times$ magnification (Zeiss, Oberkochen, Germany). The prepared *C. cayetanensis* stock was stored at 4°C . *Salmonella enterica* (ATCC 19585) was grown in nutrient broth and aliquots were stored in 20 % glycerol at -80°C . Murine norovirus 1 (MNV) was cultured in RAW 264.7 cells (Wobus et al., 2004). MNV was harvested from the lysates of infected cells after one freeze-thaw cycle, aliquoted and stored at -80°C . Salmonella stocks were enumerated by spread plating on nutrient agar and MNV stocks were enumerated by plaque assay (Cromeans et al., 2010).

Thawed water and sludge concentrates were seeded immediately prior to extraction. For each microbe stock, 10-fold serial dilutions were made to achieve concentrations close to the desired seed quantity. Dilutions were made using PBS containing 0.01 % Tween 80 and 0.001 % Antifoam Y-30 emulsion to facilitate cell dispersion. Water concentrates

were seeded with 1,000 *C. cayetanensis* oocysts, 1,000 CFU of *S. enterica*, and 1,000 PFU of MNV, regardless of input volume. Sludge concentrates were only seeded with *C. cayetanensis* oocysts (no *S. enterica* or MNV) at seed levels of either 1,000 total oocysts, regardless of extraction method input volume, or with 500 oocysts per gram input volume.

2.3. Nucleic acid extraction methods

The PowerViral and UNEX extraction methods were evaluated for tap, surface, produce wash water, and sludge samples (Table 1). The PowerSoil method was evaluated for sludge sample concentrates, which allowed for extraction of a larger sample volume (2 g vs. 0.2 g wet sludge for PowerViral and UNEX) (Table 2). Extractions were performed in triplicate for each sample type and seed level to account for potential heterogeneity within the sample matrix and for variation in the microbe seed quantity across the three replicates. For all extraction methods and sample types, an extraction blank consisting of nuclease-free water was extracted alongside samples. For a subset of extracts, nucleic acid concentration (ng/μL) and purity (A260/280 ratio) was measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions.

2.3.1. PowerViral extraction: Two hundred microliters of water concentrates and 0.2 g sludge concentrates were extracted using the AllPrep® PowerViral® DNA/RNA kit (Qiagen, Hilden, Germany) according to the manufacturers protocol with the following modifications. Dithiothreitol (DTT) (Fisher, Fair Lawn, NJ) was added to the lysing buffer at a concentration of 40 mM to reduce RNase activity instead of β-mercaptoethanol, per manufacturer's recommendation (see Supplementary Information Table S1 for comparison data). Bead beating was performed on a FastPrep-24™ Classic bead beating grinder and lysis system (MP biomedical, Irvine, CA) at a speed of 6 m/s for 60 s, and the final elution was completed with 100 μL of TE buffer.

Method variations were assessed for two sets of surface and tap water samples and one set of wash water samples (Table 1). During these experiments, an additional set of samples were extracted using input volumes of 375 μL by proportionally scaling up reagents. A separate set of samples were extracted using Lysing Matrix E (ME) 2-mL bead tubes (MP Biomedicals, Irvine, CA). The ME bead tubes contain 1.4 mm ceramic spheres, 0.1 mm silica spheres and one 4 mm glass bead, while the PowerViral bead tubes contain 0.1 mm glass beads.

2.3.2. UNEX extraction: Two hundred microliters of water concentrates and 0.2 g sludge concentrates were extracted using the UNEX method (Hill et al., 2015). Sample concentrates were added to FastPrep® 2-mL Lysing Matrix tubes (hereafter referred to as UNEX bead tubes) (MP Biomedicals, Irvine, CA) containing ~200 mg each of 0.2 μm and 0.5 μm zirconium oxide beads (Union Process, Akron, OH). Two hundred microliters of UNEX lysis buffer (Microbiologics, St. Cloud, MN) and 20 μL Proteinase K (Qiagen, Hilden, Germany) were added to the bead tubes and the contents were mixed by briefly vortexing. Samples were incubated before bead beating to allow for cell lysing and Proteinase K digestion. Initially, incubation was performed for 15 min at room temperature. However, data from the first two experiments (Surface A and B; Tap A) showed that the

UNEX method yielded lower than expected target organism recovery based on the seed level. Thereafter, the incubation conditions were adjusted to 56 °C for 1 h to attempt to improve protein digestion (Qvarnstrom et al., 2018a).

Bead beating was performed on a FastPrep-24™ Classic at a speed of 6 m/s for 60 s, followed by centrifugation at 10,000 $\times g$ for 30 s to pellet cell debris. Bead beating supernatants were added to HiBind® RNA Spin Columns (Omega BioTek, Norcross, GA) and then centrifuged at 10,000 $\times g$ for 1 min to capture DNA and RNA. The columns were washed first with 500 μ L of 100 % ethanol, then 500 μ L of 70 % ethanol, followed by centrifugation after each at 10,000 $\times g$ for 1 min. A final dry spin was performed at 10,000 $\times g$ for 1 min to remove any residual ethanol from the silica columns. Nucleic acid was eluted from the columns with 100 μ L of TE buffer by centrifuging at 9000 $\times g$ for 1 min. The eluates were passed through the OneStep PCR Inhibitor Removal Kit, following manufacturer's instructions (Zymo Research, Tustin, CA).

Method variations were assessed for two sets of surface and tap water samples and one set of wash water samples in the same manner as for PowerViral extractions (Table 1).

2.3.3. PowerSoil extraction: Two grams of sludge concentrates were extracted following the manufacturers protocol using the RNeasy PowerSoil Total RNA kit (Table 2). After the initial RNA elution, the RNeasy PowerSoil DNA Elution kit (Qiagen, Hilden, Germany) was used to extract the DNA from each sample. The final DNA and RNA pellets were each resuspended with 100 μ L TE buffer.

2.4. Molecular analysis

All nucleic acid extracts were stored at 4 °C and subjected to qPCR within 24 h of DNA extraction. qPCR was performed for the detection of *C. cayetanensis* targeting the 18S rRNA gene (Qvarnstrom et al., 2018a), for *S. enterica* targeting the ttrRSBCA locus (Malorny et al., 2004), and for MNV targeting a region of the RNA-dependent RNA polymerase gene (Hill et al., 2007). Water extracts were analyzed in duplicate for each target with 5 μ L template in 50 μ L reaction volumes. TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems, Waltham, MA) was used for the *C. cayetanensis* and *S. enterica* assays, and TaqMan™ Fast Virus 1-Step Master Mix was used for MNV assays. Bovine serum albumin (0.5 mg/mL) and T4 Gene 32 protein (gp32, New England Biolabs, Ipswich, MA) (25 μ g/mL) were added to the reaction mixture for all three targets to improve amplification efficiency and reduce inhibition effects.

qPCR was performed in a 7500 Real-Time PCR System with software version 2.3 (Applied Biosystems). Cycling conditions for *C. cayetanensis* and *S. enterica* each started with denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, then annealing and fluorescence acquisition at 67 °C for one minute for the *C. cayetanensis* assay and at 60 °C for the *S. enterica* assay. qPCR cycling conditions for MNV were one cycle of reverse transcription at 50 °C for five minutes, denaturation at 95 °C for 20 s, followed by 45 cycles of denaturation at 95 °C for 20 s, and annealing and fluorescence acquisition at 56 °C for one minute. The amplification threshold was set to 0.02 delta normalized reporter value (ΔR_n) for *C. cayetanensis*, 0.03 ΔR_n for *S. enterica*, and 0.05

for MNV. Positive controls consisted of genomic DNA or RNA of each organism, and no-template controls consisted of nuclease-free water. Extraction blanks from each extraction method, positive controls, and no-template controls were analyzed in duplicate with each instrument run.

Sludge extracts were analyzed for *C. cayetanensis* following the same qPCR assay specifications as for water samples with the following modifications. All samples and controls were analyzed in triplicate. A 1:4 dilution was analyzed in addition to each undiluted sample for PowerSoil extracts. Five hundred copies of a commercially synthesized DNA oligo was added to each qPCR as a non-competitive internal amplification control (IAC) to monitor for reaction inhibition as previously described (U.S. Food and Drug Administration, 2017). A standard calibration curve consisting of a synthetic gBlock gene fragment (IDT gBlock, Coralville, IA) was generated with dilutions ranging from 10^6 through 10^0 copies per reaction (U.S. Food and Drug Administration, 2017) and included in three instrument runs to generate a pooled standard curve equation for quantification of the *C. cayetanensis* target.

Gene targets were considered detected if amplification occurred at a quantification cycle threshold (C_q) less than 40 for both replicates for water extracts and for at least two out of three replicates for sludge extracts. Sludge extracts were scored as detected if either set of technical replicates (undilute or 1:4 dilution) met these criteria. A mean C_q of 40 was assigned to all non-detect water extracts for statistical analyses. For sludge extracts with 2 of 3 positive replicates, a C_q of 40 was assigned to the non-detect replicate and then averaged with the other two C_q values for statistical analyses. Detected *C. cayetanensis* 18S rRNA gene target copy number was calculated by inputting the mean C_q of the detected technical replicates into the pooled standard curve equation. Sludge extracts in which the IAC had a ≥ 3 C_q value difference from the no-template control were classified as inhibited but were included in analyses using data from the technical replicates (undilute or 1:4) that produced the higher number of detected gene copies.

2.5. Statistical analysis

Statistical analyses for nucleic acid concentration and purity comparisons were performed using Excel's data analysis tool. Comparisons between the PowerViral and UNEX extraction methods for water samples were made using paired two-tailed *t*-tests. One way ANOVA was performed to compare nucleic acid purity for sludge samples.

Statistical analyses for extraction method performance were performed in R version 4.2.2 (R Core Team, 2021). Multiple linear regression analysis was used to assess the impact of method variations (e.g., input volume, bead tube type, heated incubation) on microbe recovery from water samples, as measured by C_q values. Separate models were built for each combination of extraction method (PowerViral or UNEX), sample type (tap or surface water), and microbe (*C. cayetanensis*, *S. enterica*, MNV). The mean C_q of the qPCR replicates for each extract was used as the response variable and indicator variables for input volume (200 μ L vs. 375 μ L) and bead tube (Matrix E vs. PowerViral or UNEX bead tubes, respectively) were used as the predictor variables. Use of a heated incubation step was also included as a predictor in regression models for the UNEX method. Models for wash water

were not constructed for either extraction method because all but one PowerViral wash water extractions used the PowerViral bead tube and all UNEX wash water extractions utilized a heated incubation step. Accordingly, a negative regression coefficient that corresponded to an estimated mean difference in C_q that was significantly different from zero at the 5 % significance level was interpreted as evidence that the corresponding methodological variation was associated with superior microbial recovery.

Comparison of PowerViral and UNEX recovery from water samples was assessed using non-parametric Wilcoxon signed-rank tests to compare the distributions of C_q values for each microbe and water type. Method variations that were not associated with significantly different C_q values in the regression analyses were considered to perform equivalently and all data from such variations were included in the performance analysis comparison to the other extraction method. Recovery was compared within a water sample (e.g., A, B, C) when data from all equivalent method modifications were available ($N = 6$ extractions per method), and when 67 % of all extracts ($N \geq 8/12$) were classified as detects. This was done to prevent bias from non-detects or unpooled data.

Multiple linear regression was performed to determine if extraction method (PowerSoil, PowerViral, or UNEX), sludge type (REC or RAS), or the sludge sample extracted (e.g. A, B, C) impacted *C. cayetanensis* percent recovery from sludge. Comparison of the performance of the extraction methods for sludge samples was compared using percent recovery instead by comparing of C_q value because the seed level was not the same for each extraction method for sludge. Percent recovery was calculated by dividing the detected number of *C. cayetanensis* 18S rRNA gene copies by the estimated seeded gene copy number. The estimated seeded gene copy number was obtained by multiplying the number of seeded oocysts by the number of gene copies of the 18S rRNA gene per genome. Microscopy observations of the *C. cayetanensis* oocyst stock revealed exclusively unsporulated oocysts (Assurian et al., 2020a). An unsporulated *C. cayetanensis* oocyst contains two haploid genomes, containing an average of 17.5 copies of the 18S rRNA gene. Therefore, the 1000-oocyst seed was estimated to contain 35,000 gene copies and the 100-oocyst seed was estimated to contain 3500 gene copies.

3. Results

3.1. Effect of input volume and bead beating method on PowerViral performance for water samples

The PowerViral extraction method resulted in consistent detection of *C. cayetanensis*, *S. enterica*, and MNV from all water sample types and method variations (Table 3, Table S2). Neither the input volume nor the bead type affected the C_q values for *S. enterica* or MNV in any water type.

C. cayetanensis recovery from tap water using the PowerViral method was impacted by input volume and bead type. Mean C_q was 3.66 cycles lower when input volume was 200 μ L versus 375 μ L (i.e., approximately $1 - \log_{10}$ more target copies recovered with 200 μ L input) [(95 % confidence interval (95 %CI): 2.21–5.11 cycles; $p < 0.001$)]. Mean C_q was 2.06

cycles lower when bead beating was done with the Matrix E bead tube versus the PowerViral bead tube (95 %CI: 0.61–3.51 cycles; $p = 0.01$).

C. cayetanensis recovery from surface water using the PowerViral method was also impacted by input volume, but not bead type. Mean C_q was 1.6 cycles lower when input volume was 200 μ L versus 375 μ L (95 % CI: 0.32–2.88 cycles; $p < 0.05$). However, mean C_q values for the PowerViral and Matrix E bead tubes were not significantly different ($p = 0.93$). All positive controls, no-template controls, and extraction blanks performed as expected.

3.2. Effect of input volume and bead beating method on UNEX performance for water samples

The UNEX extraction method resulted in inconsistent detection across water types. Detection rates for all microbes were higher for tap and wash water samples than for surface water, which had few detections (Table 3, Table S3). Analyses for the UNEX method could only be performed for tap water, as there were too few detections from surface water. Mean *S. enterica* C_q values were not impacted by input volume ($p = 0.57$), bead tube ($p = 0.06$), or heated incubation ($p = 0.96$).

C. cayetanensis recovery using the UNEX method was not impacted by input volume, as the mean C_q values for the 200 μ L and 375 μ L input volumes were not significantly different ($p = 0.98$). However, recovery was greater for both the UNEX bead tube and the heated incubation. Mean C_q was 4.18 cycles lower when bead beating was done with the UNEX bead tube versus the Matrix E tube (95 %CI: 2.67–5.69 cycles); $p < 0.001$) and was 2.57 cycles lower when extracted after a heated incubation (95 %CI: 1.33–3.80 cycles; $p < 0.001$).

MNV recovery using the UNEX method was not impacted by input volume ($p = 0.22$) or bead type ($p = 0.48$). However, a heated incubation significantly improved MNV recovery from tap water, resulting in mean C_q values that were 7.56 cycles lower than samples without heated incubation (i.e., over 2- $\log_{10}$ more target copies recovered using heated incubation) (95 %CI: 6.37–8.75 cycles; $p < 0.001$). All positive controls, no-template controls, and extraction blanks performed as expected.

3.3. Comparison of extraction method detection rates for water samples

PowerViral and UNEX method performance were compared using data from the best performing extractions for each method (Sections 3.1 and 3.2). Data from the method variations that performed equivalently were pooled (Table 3, gray shading). The PowerViral data set comprised extractions with a 200 μ L input volume and both bead tubes. The UNEX data set comprised extractions with 200 μ L and 375 μ L input volumes, a heated incubation step, and the UNEX bead tube. From these pooled data sets, *C. cayetanensis*, *S. enterica*, and MNV were detected in nearly 100 % of surface water extracts using the PowerViral method (17/18 *S. enterica* detections), but there were no detections for any microbe using the UNEX method. Almost all microbes were detected in all tap water extracts for both the PowerViral and UNEX methods except for *S. enterica* in one 200 μ L UNEX extraction. *C. cayetanensis* was detected in 100 % of wash water samples using both the UNEX and

PowerViral methods. *S. enterica* was detected in 83 % of PowerViral extracts and 100 % of UNEX extracts for wash water. MNV was detected in 100 % of wash water samples using the PowerViral method, but only 56 % of samples using the UNEX method.

3.4. Comparison of extraction method recovery for water samples

Recovery performance between the PowerViral and UNEX methods were compared using the median C_q achieved by each method (Table 4). Extracts from samples Tap B and Wash A were the only samples to meet the inclusion criteria for comparison of recovery between the PowerViral and UNEX methods. Recovery of *C. cayetanensis* was not significantly different between the methods for tap water ($W = 8.5$, $p = 0.15$) or wash water ($W = 7$, $p = 0.09$). MNV recovery was greater using the PowerViral method, as median MNV C_q values were significantly lower for tap water ($W = 0$, $p = 0.002$) and wash water ($W = 0$, $p = 0.005$) samples extracted with the PowerViral method than those extracted with the UNEX method. Analyses for *S. enterica* could only be completed for tap water samples, for which recovery was greater using the PowerViral method, which had significantly lower median C_q value than UNEX extractions ($W = 0$, $p = 0.002$).

3.5. Comparison of extraction method nucleic acid concentration and purity

Water nucleic acid concentrations (ng/ μ L dsDNA) and purity ratios (260/280 nm absorbance ratio) from 200 μ L extractions using the method-specific bead tubes are presented in Table S4. UNEX extract DNA concentrations were 12 times higher than PowerViral extracts for surface water ($p = 0.04$), and 19 times higher for tap water ($p = 0.01$). PowerViral extract purity ratios were closer to pure DNA than UNEX extracts, as measured by the deviation from the 1.8, which is considered a pure nucleic acid purity ratio. Mean purity ratio difference from 1.8 was 2.4 times higher for UNEX extracts (mean ratio difference = 1.0, SD = 0.05) than for PowerViral extracts (mean ratio difference = 0.43, SD = 0.11) for surface water ($p = 0.009$), and 2.2 times higher for tap water (UNEX: mean ratio difference = 0.97, SD = 0.04; PowerViral mean ratio difference = 0.45, SD = 0.06); $p = 0.001$).

3.6. Comparison of extraction method detection rates and inhibition for sewage sludge

C. cayetanensis was detected in 100 % of sludge extracts using the UNEX and PowerViral methods, regardless of seed quantity (Table 5). Using the PowerSoil method, in which samples were seeded with 1000 oocysts, *C. cayetanensis* was detected in all REC sludge extracts and 89 % of RAS extracts. Mean *C. cayetanensis* percent recoveries from sludge samples were 17–36 % using the PowerViral method and 4–12 % using the UNEX method (Table 5). Mean *C. cayetanensis* recoveries for the PowerSoil method were ≤ 1 %. Inhibition from RAS sludge was observed in 11–44 % of PowerViral extracts, 22–33 % of UNEX extracts, and 84 % of PowerSoil extracts (Table 5). While the percentage of inhibited extracts from REC sludge was 0–1 % for the PowerViral and UNEX methods, 58 % of PowerSoil extracts exhibited inhibition. The mean IAC C_q differences for inhibited extracts are also presented in Table 5.

3.7. Comparison of extraction method recovery for sewage sludge

Detected copies of the 18S rRNA gene were 2.43 log₁₀ copies per oocyst seeded greater when extracting with the PowerViral method versus the PowerSoil method ($p < 0.001$) and were 2.02 log₁₀ copies per oocyst seeded greater when extracting with the UNEX method versus the PowerSoil method ($p < 0.001$). Neither the type of sludge (REC or RAS) nor the sample extracted (e.g., A, B, C) were significant predictors of detected gene copies per oocyst seeded ($p > 0.05$). All no-template controls and extraction blanks performed as expected. The *C. cayetanensis* pooled standard curve slope was -3.50 , intercept was 38.0 , and R^2 was 0.998 .

3.8. Comparison of extraction method nucleic acid concentration and purity

Sludge extract nucleic acid concentrations and purity ratios are presented in Table S5. For REC sludge, one-way ANOVA demonstrated that DNA purity was significantly different for at least one of the extraction methods ($p = 0.0001$). For RAS sludge, there was no significant difference in the deviation from pure DNA by extraction method ($p = 0.09$). Statistical comparisons of nucleic acid concentration were not performed, as the input amounts for the extraction methods were not equivalent for all three extraction methods.

4. Discussion

The PowerViral and UNEX extraction methods resulted in similar recovery of *C. cayetanensis* nucleic acid in tap and wash water, while the PowerViral method resulted in greater recovery from surface water. However, the PowerViral extraction method resulted in greater recovery of *S. enterica* and MNV than the UNEX extraction method across all water types. For sludge samples, both the PowerViral and UNEX resulted in better recovery of *C. cayetanensis* than the PowerSoil method. Several factors could have contributed to the performance differences between methods, including microbe characteristics, sample type, and efficacy of cell lysis and inhibitor removal.

Extraction recovery was highly variable based on sample type, with surface water proving the greatest challenge. We evaluated method robustness for water samples in two ways. The first was simply evaluating different types of water samples with different type and levels of organic matter and potential inhibitory substances. The second was adding organic matter by increasing the input volume from 200 μL to 375 μL . While PowerViral provided consistent detection of all microbes, *C. cayetanensis* recovery from surface and tap water was lower with the 375 μL input volume. This suggests that the PowerViral bead beating process may have been hindered by the additional organic matter or that the additional volume adversely affected the bead to sample ratio. It is important to note that the manufacturer-specified input volume is 200 μL . The PowerViral volumes and processes were modified accordingly for the 375 μL input volume, but additional beads were not added.

UNEX method recovery was greatly affected by the sample matrix, with consistent detection of all microbes in tap water, inconsistent detection of MNV in wash water, and no detections of any of the microbes in surface water. Although the larger 375 μL input volume had no effect on UNEX recovery for tap water, we did not study the effect of scaling up the

UNEX to the designed maximum input volume of 750 μ L. The overall performance of the UNEX method suggests it may be most effective for use with low inhibitory substance water matrices such as potable water.

Varying recovery between different cell lysis methods could also explain some of the performance differences between the methods. Cell lysis was facilitated by lysis buffer, heat (for UNEX), and bead beating. Bead beating for the UNEX and PowerViral extractions was performed on the FastPrep instrument, ensuring the same bead beating force for these extractions. However, bead beating for the PowerSoil method was performed on a vortexer instead of a bead beater. The poor recovery of *C. cayetanensis* using the PowerSoil method compared to the UNEX and PowerViral methods highlight the importance of bead beating for these organisms. The lower shearing force of the vortexer compared to a bead beater could have resulted in incomplete lysis of the *C. cayetanensis* oocysts. For bacteria and viruses, lysis buffer alone may be sufficient to lyse cells and virions, but physical disruption by bead beating is critical for chemically tolerant organisms such as *C. cayetanensis* (Cama and Ortega, 2024; Qvarnstrom et al., 2018a).

Extraction performance for sludge samples varied greatly by extraction method and sample type. While detection rates were nearly 100 % for all extraction methods, the percent recovery of the PowerSoil method was markedly lower than either the PowerViral or UNEX methods. The RAS sample matrix, with a higher proportion of organic matter, presented a greater challenge to the PowerSoil kit than the other extraction methods. Mean PowerSoil percent recovery from RAS sludge was 6-fold lower than for REC sludge.

The poor performance of the PowerSoil method may be due, in part, to ineffective removal of qPCR inhibitors. The PowerSoil method produced a higher proportion of inhibited extracts than the PowerViral and UNEX methods. The PowerViral and PowerSoil methods utilize the same flocculation procedure for inhibitor removal (Braid et al., 2003). In contrast the UNEX inhibitor removal process utilizes polyvinylpyrrolidone (PVPP) to bind humic acids as nucleic acid is passed through the PVPP columns. Despite the differences between the PowerViral and UNEX methods, our results demonstrate that each of these methods adequately remove inhibiting substances from sludge samples. This study was subject to several limitations. The microbial seed quantities were not directly quantified. Instead, seed quantities were calculated mathematically based on the dilution factors of the microbial stock concentrations. Therefore, differences in performance between methods could have been due, in part, to variations in seed quantities between the extracts. Extractions were performed in triplicate to help overcome this potential limitation, and our data show low C_q standard deviation values, which suggests that differences in method performance are not likely due to seeding variation. This was a small study and the ability to do statistical analyses was limited in some cases. The impact of inhibition on the qPCR results for water sample extracts could not be assessed, as IACs were not included in water extract qPCRs. While IACs provided data on how inhibitory the sludge extracts were to qPCR, it was not possible to assess how the individual aspects of the extraction processes facilitated or hindered nucleic acid recovery and inhibitor removal (e.g. filter clogging limiting DNA binding, insufficient elution from filter, inhibitors overwhelmed removal system, etc.).

5. Conclusions

Data from this study can help inform selection of extraction methods for use in the detection of food safety related microbes from environmental samples including tap water, irrigation water, and sewage sludge. The following conclusions from this study can be used to inform method selection:

- The PowerViral extraction method resulted in consistent detection across water sample types.
- The UNEX extraction method resulted in inconsistent detection across water types.
- Surface water presented the greatest challenge to UNEX performance.
- Bead beating is critical for recovery of *C. cayetanensis*, and vortex-beating may not be sufficient to lyse oocysts.
- A heated lysis buffer incubation may improve UNEX recovery for some organisms.
- The method-specified bead beating beads performed as well or better than alternative bead types.
- The PowerViral and UNEX methods outperformed the PowerSoil method for sludge samples.
- Nucleic acid purity was a better indicator of method performance than yield for water samples, but there was no correlation between purity or yield and method performance for sludge samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank David Holcomb for assistance with statistical analyses and manuscript review. The use of trade names and names of commercial sources is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention or the U.S. Department of Health and Human Services. The findings and conclusions are those of the authors and do not necessarily represent those of the Centers for Disease Control and Prevention.

Funding

This research received no external funding.

Data availability

Data will be made available on request.

References

Abanyie F, Harvey RR, Harris JR, Wiegand RE, Gaul L, Desvignes-Kendrick M, Irvin K, Williams I, Hall RL, Herwaldt B, Gray EB, Qvarnstrom Y, Wise ME, Cantu V, Cantey PT, Bosch S, DA Silva AJ, Fields A, Bishop H, Multistate Cyclosporiasis Outbreak Investigation Team, 2015. 2013

- multistate outbreaks of *Cyclospora cayetanensis* infections associated with fresh produce: focus on the Texas investigations. *Epidemiol. Infect* 143 (16), 3451–3458. 10.1017/S0950268815000370. [PubMed: 25865140]
- Abbaszadegan M, Huber MS, Gerba CP, Pepper IL, 1993. Detection of enteroviruses in groundwater with the polymerase chain reaction. *Appl. Environ. Microbiol.* 59 (5), 1318–1324. 10.1128/aem.59.5.1318-1324.1993. [PubMed: 7685998]
- Almeria S, da Silva AJ, Blessington T, Cloyd TC, Cinar HN, Durigan M, Murphy HR, 2018. Evaluation of the U.S. Food and Drug Administration validated method for detection of *Cyclospora cayetanensis* in high-risk fresh produce matrices and a method modification for a prepared dish. *Food Microbiol.* 76, 497–503. 10.1016/j.fm.2018.07.013. [PubMed: 30166179]
- Assurian A, Murphy H, Ewing L, Cinar HN, da Silva A, Almeria S, 2020a. Evaluation of the U.S. Food and Drug Administration validated molecular method for detection of *Cyclospora cayetanensis* oocysts on fresh and frozen berries. *Food Microbiol* 87, 103397. 10.1016/j.fm.2019.103397. [PubMed: 31948637]
- Assurian A, Murphy H, Shipley A, Cinar HN, Silva DA, A., & Almeria S, 2020b. Assessment of commercial DNA cleanup kits for elimination of real-time PCR inhibitors in the detection of *Cyclospora cayetanensis* in cilantro. *J. Food Prot* 83 (11), 1863–1870. 10.4315/JFP-20-139. [PubMed: 32722800]
- Borchardt MA, Spencer SK, Bertz PD, Ware MW, Dubey JP, Alan Lindquist HD, 2009. Concentrating toxoplasma gondii and *Cyclospora cayetanensis* from surface water and drinking water by continuous separation channel centrifugation. *J. Appl. Microbiol* 107 (4), 1089–1097. 10.1111/j.1365-2672.2009.04316.x. [PubMed: 19486387]
- Braid MD, Daniels LM, Kitts CL, 2003. Removal of PCR inhibitors from soil DNA by chemical flocculation. *J. Microbiol. Methods* 52 (3), 389–393. 10.1016/s0167-7012(02)00210-5. [PubMed: 12531508]
- Cama VA, Ortega YR, 2024. *Cyclospora cayetanensis*. In: Smithers GW (Ed.), *Encyclopedia of Food Safety*, Second edition. Academic Press, pp. 458–474. 10.1016/B978-0-12-822521-9.00054-X.
- Centers for Disease Control and Prevention, 2021. CDC – Cyclosporiasis. Parasites-Cyclosporiasis. January 19 <https://www.cdc.gov/parasites/cyclosporiasis/index.html>.
- Cromeans TL, Kahler AM, Hill VR, 2010. Inactivation of adenoviruses, enteroviruses, and murine norovirus in water by free chlorine and monochloramine. *Appl. Environ. Microbiol* 76 (4), 1028–1033. 10.1128/AEM.01342-09. [PubMed: 20023080]
- Erickson MC, Ortega YR, 2006. Inactivation of protozoan parasites in food, water, and environmental systems. *J. Food Prot* 69 (11), 2786–2808. 10.4315/0362-028X-69.11.2786. [PubMed: 17133829]
- Gentry-Shields J, Wang A, Cory RM, Stewart JR, 2013. Determination of specific types and relative levels of QPCR inhibitors in environmental water samples using excitation–emission matrix spectroscopy and PARAFAC. *Water Res* 47 (10), 3467–3476. 10.1016/j.watres.2013.03.049. [PubMed: 23601829]
- Hadjilouka A, Tsaltas D, 2020. *Cyclospora cayetanensis*—major outbreaks from ready to eat fresh fruits and vegetables. *Foods* 9 (11), 1703. 10.3390/foods9111703. [PubMed: 33233660]
- Hill VR, Kahler AM, Jothikumar N, Johnson TB, Hahn D, Cromeans TL, 2007. Multistate evaluation of an ultrafiltration-based procedure for simultaneous recovery of enteric microbes in 100-liter tap water samples. *Appl. Environ. Microbiol* 73 (13), 4218–4225. 10.1128/AEM.02713-06. [PubMed: 17483281]
- Hill VR, Narayanan J, Gallen RR, Ferdinand KL, Cromeans T, Vinjé J, 2015. Development of a nucleic acid extraction procedure for simultaneous recovery of DNA and RNA from diverse microbes in water. *Pathogens* 4 (2). 10.3390/pathogens4020335. Article 2.
- Iker BC, Bright KR, Pepper IL, Gerba CP, Kitajima M, 2013. Evaluation of commercial kits for the extraction and purification of viral nucleic acids from environmental and fecal samples. *J. Virol. Methods* 191 (1), 24–30. 10.1016/j.jviromet.2013.03.011. [PubMed: 23578704]
- Kahler AM, Johnson TB, Hahn D, Narayanan J, Derado G, Hill VR, 2015. Evaluation of an ultrafiltration-based procedure for simultaneous recovery of diverse microbes in source waters. *Water* 7 (3), 1202–1216. 10.3390/w7031202. [PubMed: 26530003]

- Kahler AM, Mattioli MC, da Silva AJ, Hill V, 2021. Detection of *Cyclospora cayetanensis* in produce irrigation and wash water using large-volume sampling techniques. *Food and Waterborne Parasitology* 22. 10.1016/j.fawpar.2021.e00110.
- Kahler AM, Hofstetter J, Arrowood M, Peterson A, Jacobson D, Barratt J, da Silva ALBR, Rodrigues C, Mattioli MC, 2024. Sources and prevalence of *Cyclospora cayetanensis* in southeastern U.S. growing environments. *J. Food Prot* 87 (7), 100309. 10.1016/j.jfp.2024.100309. [PubMed: 38815808]
- Malorny B, Paccassoni E, Fach P, Bunge C, Martin A, Helmuth R, 2004. Diagnostic real-time PCR for detection of *Salmonella* in food. *Appl. Environ. Microbiol* 70 (12), 7046–7052. 10.1128/AEM.70.12.7046-7052.2004. [PubMed: 15574899]
- O'Brien M, Rundell ZC, Nemec MD, Langan LM, Back JA, Lugo JN, 2021. A comparison of four commercially available RNA extraction kits for wastewater surveillance of SARS-CoV-2 in a college population. *Sci. Total Environ* 801, 149595. 10.1016/j.scitotenv.2021.149595. [PubMed: 34418621]
- Quintero-Betancourt W, Peele ER, Rose JB, 2002. *Cryptosporidium parvum* and *Cyclospora cayetanensis*: A review of laboratory methods for detection of these waterborne parasites. *J. Microbiol. Methods* 49 (3), 209–224. 10.1016/S0167-7012(02)00007-6. [PubMed: 11869786]
- Qvarnstrom Y, Benedict T, Marcet PL, Wiegand RE, Herwaldt BL, da Silva AJ, 2018a. Molecular detection of *Cyclospora cayetanensis* in human stool specimens using UNEX-based DNA extraction and real-time PCR. *Parasitology* 145 (7), 865–870. 10.1017/S0031182017001925. [PubMed: 29113617]
- Qvarnstrom Y, Wei-Pridgeon Y, Van Roey E, Park S, Srinivasamoorthy G, Nascimento FS, Moss DM, Talundzic E, Arrowood MJ, 2018b. Purification of *Cyclospora cayetanensis* oocysts obtained from human stool specimens for whole genome sequencing. *Gut Pathogens* 10, 45. 10.1186/s13099-018-0272-7. [PubMed: 30337964]
- R Core Team, 2021. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. <https://www.R-project.org/>.
- Shields JM, Olson BH, 2003. PCR-restriction fragment length polymorphism method for detection of *Cyclospora cayetanensis* in environmental waters without microscopic confirmation. *Appl. Environ. Microbiol.* 69 (8), 4662–4669. 10.1128/AEM.69.8.4662-4669.2003. [PubMed: 12902255]
- Shields JM, Joo J, Kim R, Murphy HR, 2013. Assessment of three commercial DNA extraction kits and a laboratory-developed method for detecting *Cryptosporidium* and *Cyclospora* in raspberry wash, basil wash and pesto. *J. Microbiol. Methods* 92 (1), 51–58. 10.1016/j.mimet.2012.11.001. [PubMed: 23147278]
- Smith HV, Paton CA, Mitambo MM, Girdwood RW, 1997. Sporulation of *Cyclospora* sp. Oocysts. *Applied and Environmental Microbiology* 63 (4), 1631–1632. [PubMed: 9097458]
- Temesgen TT, Barlaam A, Tysnes KR, Robertson LJ, 2020. Comparative evaluation of UNEX-based DNA extraction for molecular detection of *Cyclospora cayetanensis*, *Toxoplasma gondii*, and *Cryptosporidium parvum* as contaminants of berries. *Food Microbiol* 89, 103447. 10.1016/j.fm.2020.103447. [PubMed: 32138995]
- Thatcher SA, 2015. DNA/RNA preparation for molecular detection. *Clin. Chem* 61 (1), 89–99. 10.1373/clinchem.2014.221374. [PubMed: 25451869]
- Totton SC, O'Connor AM, Naganathan T, Martinez BAF, Vriezen ER, Torrence ME, Sargeant JM, 2021. A scoping review of the detection, epidemiology and control of *Cyclospora cayetanensis* with an emphasis on produce, water and soil. *Epidemiology & Infection* 149. 10.1017/S0950268821000200.
- Tsai YL, Olson BH, 1992. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. *Appl. Environ. Microbiol* 58 (7), 2292–2295. 10.1128/aem.58.7.2292-2295.1992. [PubMed: 1386212]
- U.S Food and Drug Administration, 2020. Outbreak Investigation of *Cyclospora*: Bagged Salads (June 2020). FDA. <https://www.fda.gov/food/outbreaks-foodborne-illness/outbreak-investigation-cyclospora-bagged-salads-june-2020>.

- U.S Food and Drug Administration, 2021, July 1. Cyclospora Prevention, Response and Research Action Plan. Foodborne Pathogens. FDA. <https://www.fda.gov/food/foodborne-pathogens/cyclospora-prevention-response-and-research-action-plan>.
- U.S. Food and Drug Administration, 2017. BAM Chapter 19b: Molecular Detection of Cyclospora cayetanensis in Fresh Produce Using Real-Time PCR. In Bacteriological Analytical Manual (8th ed.). FDA.
- Wobus CE, Karst SM, Thackray LB, Chang K-O, Sosnovtsev SV, Belliot G, Krug A, Mackenzie JM, Green KY, Iv HWV, 2004. Replication of norovirus in cell culture reveals a tropism for dendritic cells and macrophages. PLoS Biol 2 (12), e432. 10.1371/journal.pbio.0020432. [PubMed: 15562321]
- Zielińska S, Radkowski P, Blendowska A, Ludwig-Gałęzowska A, Łoś JM, Łoś M, 2017. The choice of the DNA extraction method may influence the outcome of the soil microbial community structure analysis. MicrobiologyOpen 6 (4), e00453. 10.1002/mbo3.453. [PubMed: 28217901]

Table 1

Number of replicate water sample extractions using the PowerViral and UNEX methods using different input volumes, bead beating bead composition (bead tube), and UNEX lysis buffer incubation conditions.

Extraction method		PowerViral			UNEX		
Input volume (µL)		200	375		200	375	
Bead tube ^a		PV	ME		UNEX	ME	UNEX
Heated incubation (Y/N) ^b		N	N		Y	Y	N
Water type	Sample	No. of replicate extractions					
		A	3	3	3	-	3
Surface	A	3	3	3	3	-	3
	B	3	3	3	3	-	3
	C	3	-	3	-	-	-
	D	3	-	3	-	-	-
Tap	A	3	3	3	3	-	3
	B	3	3	3	3	-	3
Wash	A	3	3	3	3	-	3
	B	3	-	3	-	-	-

^a PV = bead tube provided in PowerViral kit; ME = Matrix E tube; UNEX = bead tube indicated for UNEX method.

^b Heated incubation of lysis buffer prior to bead beating. Y: Yes; N: No.

^c -: no extractions conducted for these conditions.

Table 2

Number of replicate sludge sample extractions using the PowerViral, UNEX, and PowerSoil methods with different *C. cayetanensis* seed quantities.

Extraction method		PowerViral		UNEX		PowerSoil
Input amount		0.2mL		0.2mL		2g
Oocyst seed quantity		100	1000	100	1000	1000
Sludge type	Sample	No. of replicate extractions				
RAS ^a	A	3	3	3	3	6
	B	3	3	3	3	6
	C	3	3	3	3	6
REC ^b	A	3	3	3	3	3
	B	3	3	3	3	6
	C	3	3	3	3	3

^aRAS: return activated sludge from aeration basin.

^bREC: thickener recovery system sludge.

Table 3

Number of replicate extract detections from water samples seeded with *C. cayetanensis*, *S. enterica*, and MNV using the PowerViral and UNEX methods using different input volumes, bead beating bead composition (bead tube), and UNEX lysis buffer incubation conditions. Gray shading indicates the method variations that performed equivalently for each extraction method and only these data were used for analyses between methods.

Extraction method		Power Viral				UNEX					
Input volume (µL) Bead tube ^a Heated incubation (Y/N) ^b		200		375		200		375		UNEX	
		PV	ME	PV	ME	UNEX	ME	UNEX	ME	Y	N
		N	N	N	N	Y	Y	N	N	Y	N
Water type	Sample	C. cayetanensis detections (N=3)									
Surface	A	3	3	3	3	- ^c	0	-	0	-	0
	B	3	3	3	3	-	0	-	0	-	1
	C	3	3	-	-	0	-	-	-	-	-
	D	3	-	-	-	0	-	-	-	-	-
Tap	A	3	3	3	2	-	3	-	1	-	3
	B	3	3	3	3	-	-	3	-	3	-
Wash	A	3	3	3	3	3	-	3	-	3	-
	B	3	-	-	-	-	-	-	-	-	-
Water type	Sample	S. enterica detections (N=3)									
Surface	A	3	3	3	3	-	0	-	0	-	0
	B	3	3	2	2	-	0	-	0	-	1
	C	3	3	-	-	0	-	-	-	-	-
	D	3	-	-	-	0	-	-	-	-	-
Tap	A	3	3	3	3	-	2	-	1	-	2
	B	3	3	3	3	2	-	0	-	3	-
Wash	A	ND ^d	2	3	3	ND	-	2	-	3	-
	B	3	-	-	-	3	-	-	-	-	-
Water type	Sample	MNV detections (N=3)									
Surface	A	-	-	-	-	-	-	-	-	-	-
	B	3	3	3	3	-	0	-	0	-	0
	C	3	-	-	-	0	-	-	-	-	-
	D	3	-	-	-	0	-	-	-	-	-
Tap	A	3	3	3	3	-	2	-	2	-	3
	B	3	3	3	3	3	-	3	-	3	-
Wash	A	3	3	3	3	1	-	2	-	1	-
	B	3	-	-	-	3	-	-	-	-	-

^apV = bead tube provided in Power Viral kit; ME = Matrix E tube; UNEX = bead tube indicated for UNEX method.

^bHeated incubation of lysis buffer prior to bead beating. Y: Yes; N: No.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

ND: no data obtained due to a seeding error for these replicates.
c; no extractions conducted for these conditions.

Table 4

Median and range of the mean C_q values for *C. cayetanensis*, *S. enterica* and MNV across extraction replicates for each water sample (Tap B and Wash A) using PowerViral and UNEX methods.

Organism	Sample	Method ^a	Median C _q Value (range)
<i>C. cayetanensis</i>	Tap B	PowerViral	31.23 (30.54–31.73)
		UNEX	31.66 (30.77–32.79)
	Wash A	PowerViral	32.63 (31.74–34.42)
		UNEX	33.95 (33.01–34.39)
<i>S. enterica</i>	Tap B	PowerViral	32.06 (31.67–32.47)
		UNEX	33.27 (32.61–37.68)
MNV	Tap B	PowerViral	26.86 (26.20–27.09)
		UNEX	29.90 (29.30–30.24)
	Wash A	PowerViral	27.96 (27.81–28.12)
		UNEX	35.44 (34.65–40)

^aThe PowerViral category included all samples with a 200 µL input volume that were extracted with either the PowerViral or Matrix E bead tube. The UNEX category included all samples with either a 200 µL or 375 µL input volume extracted with the UNEX bead tube and a heated incubation.

Table 5

Number of replicate extract *C. cayetanensis* detections from sludge samples, mean percent recovery and standard deviation, number of inhibited extracts, and mean difference in internal amplification control C_q values for inhibited samples.

Sludge type	Extraction method	Seed quantity	N	Detects	Mean % recovery (SDV)	Inhibited extracts (%)	Mean IAC C _q difference for inhibited extracts
RAS	PowerSoil	1000	18	16	0.17 (0.13)	15 (83)	6.65
	PowerViral	1000	9	9	28 (5)	1 (11)	3.16
	PowerViral	100	9	9	17 (13)	4 (44)	6.42
	UNEX	1000	9	9	10 (2)	2 (22)	3.32
	UNEX	100	9	9	4 (3)	3 (33)	5.85
REC	PowerSoil	1000	12	12	1.1 (0.6)	7 (58)	5.07
	PowerViral	1000	9	9	32 (7)	0	NA ^a
	PowerViral	100	9	9	36 (9)	0	NA
	UNEX	1000	9	9	11 (3)	0	NA
	UNEX	100	9	9	12 (4)	1 (11)	3.12

^aNA: not applicable; no inhibition observed in these extracts.