Detection and Quantification of *Cryptosporidium parvum* in Natural Soil Matrices and Leachates Using qPCR

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THESIS

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To my parents,
Ayşegül and Veli KÖKEN

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
C. parvum	*Cryptosporidium parvum*
Ct	Threshold Cycle
DI	De-Ionized
EDTA	Ethylenediaminetetraacetic acid
IMS	Immunomagnetic Separation
PEG	Polyethylene Glycol
PR	Percentage of Recovery
SDS	Sodium Dodecyl Sulfate
TE	Tris-EDTA

SUMMARY

Detection and quantification of *Cryptosporidium parvum* oocysts in natural soil matrix and leachates are critical to elucidate the fate and transport of *C. parvum* in the nature, assess the risk of contamination of water resources and potential threat to public health. Traditional detection and quantification methods are time-consuming, labor-intensive, lack of sensitivity and specificity. Real-Time Quantitative Polymerase Chain Reaction (qPCR) overcomes the limitations in traditional methods and produces sensitive and accurate detections of small numbers of *C. parvum*. A few qPCR studies were present in the literature for detection and quantification of *C. parvum* in environmental water samples; however *C. parvum* in natural soil matrices and leachates have not been researched yet.

This research was focused on developing a qPCR protocol for sensitive and specific detection and quantification of *C. parvum* in natural soil matrices and leachates. The physicochemical parameters—lysis media (TE buffer, Chelex-100), number of thermal shocks (5, 10 or 15) and thawing temperatures (37, 65 or 95^{o}C)—controlling the DNA extraction efficiency were investigated. The oocyst age was found to be the most critical parameter affecting the oocyst disruption and it was found to be less dependent when oocysts were disrupted in TE than in Chelex-100 buffer. Changes in thawing temperature and number of thermal shocks were found more remarkable in aged than in young oocysts and increasing the number of thermal shocks beyond five did not improve the oocyst disruption. The most efficient oocyst disruption method for *C. parvum* oocysts regardless of their age was then established as 5 thermal shocks with thawing at 65^{o}C in TE buffer. The DNA extraction method was coupled

with purification columns to remove PCR inhibitors in environmental samples and additional PCR facilitators—$MgCl_2$ and BSA—were examined to remove residual inhibitors and improve the amplicon yield. The combination of 3 mM $MgCl_2$ and 600 ng/µl BSA yielded highest amplicon yield for both young and aged oocysts. The optimized parameters of DNA extraction and qPCR assay provided very specific and sensitive detections of *C. parvum* in our study. The minimum detection limit was 0.667 for young and 6.67 for aged *C. parvum* per PCR reaction and the accuracy of the detections and quantifications were 0.999. The performance of developed protocol was further tested in contrasting soil and leachate samples on the basis of PR values. Mean PR values were calculated as 40±20% Trenton, Greenson and Sparta soil leachates and 43±13% for DI water. Sucrose flotation was determined as a better isolation method than two-phase flotation and it was further experimented for varying concentrations of *C. parvum.* The lowest and highest PR values were determined as 4.3% and 107.8%, respectively.

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1. INTRODUCTION

Cryptosporidium parvum is a protozoan parasite that causes Cryptosporidiosis—an intestinal disease. It can be transmitted between humans, between animals, and between species. Numerous Cryptosporidiosis outbreaks have been reported worldwide (Smith and Rose, 1998). Both humans and animals may be exposed to *C. parvum* through consumption of contaminated water and food as well as direct contact with contaminated soil and infected hosts (Fayer, 2004; Zilberman et al., 2009). The median infection dose of *C. parvum* in humans was established to range from 87 to 132 ingested oocysts and to be impacted by the age and the immune system condition of the individual; it has also been reported that as few as 9 oocysts may infect humans (Fayer et al., 2000; Xiao et al., 2004).

C. parvum is released into the environment in the fecal materials of infected hosts under the form of oocysts and remains potentially infectious for several months depending on the environmental conditions and stresses it encounters (Fayer, 2004). Infected hosts may excrete *C. parvum* oocysts directly to soil and surface water. Application of manure and irrigation with untreated effluents of agricultural fields may also contribute to the prevalence of *C. parvum* oocysts in the environment (Zilberman et al., 2009). Research studies have demonstrated that *C. parvum* oocysts that are deposited on the soil surface may be transported to surface water by runoff (Chacin-Bonilla et al., 2008) and to groundwater by infiltration (Mawdsley et al., 1996; Smith and Rose 1998: Darnault et al., 2003; 2004).

The isolation, detection, and quantification of *C. parvum* in the environment are complex because *C. parvum* oocysts may be associated with natural materials. Efficient isolation, purification, and concentration techniques are necessary for environmental detection and quantification of *C. parvum* oocysts (Chestnot and Schwartzbrod, 2004). Isolation techniques include density gradient flotation and filtration (Morgan and Thompson, 1998). Immunonological assays followed by microscopic examinations are commonly applied methods in detection and quantification of *C. parvum* in environmental samples; however the sensitivity and specificity of these methods are limited (Morgan and Thompson, 1998; Jex et al., 2008; Jothikumar et al., 2008).

Alternative molecular detection methods like PCR have been used for detection of *C. parvum* to overcome limitations in traditional detection methods (Johnson et al., 1995). PCR-based methods are highly sensitive and specific for detection of small numbers of *C. parvum*, and they are time- and cost-effective (Morgan and Thompson, 1998; Nichols and Smith, 2004). In traditional PCR, a particular sequence of DNA fragment is amplified by consecutive heating and cooling cycles, and high copies of PCR products (amplicons) are generated in a thermocycler. Unlike traditional PCR, in qPCR the amount of generated amplicons is measured as it occurs, enabling direct quantification without post-PCR analysis.

The amount and quality of extracted DNA directly affects the PCR results, particularly in identification and quantification of small numbers of *C. parvum* in environmental samples (Nichols and Smith, 2003). DNA of *C. parvum* may be extracted using commercial DNA

extraction kits (Guy et al., 2003; Jiang et al., 2005; Jothikumar et al., 2008), IMS (Deng et al. 1997; Fontaine and Guillot, 2003) or physical/chemical treatments (Fontaine and Guillot, 2002; Nichols and Smith, 2004). The application of commercial DNA extraction kits and IMS to environmental samples may be limited due to their costs and the possible interaction of reagents with natural materials (Morgan and Thompson, 1998; Lowery et al. 2000; Chesnot and Schwartzbrod, 2004; Jiang et al. 2005; Ramirez and Sreevatsan, 2006). Disrupting *C. parvum* oocysts in a lysis media by consecutive thermal shocks (freeze-thaw cycles) is commonly applied DNA extraction method (Johnson, et al. 1995; Fontaine and Guillot, 2002; Monis et al., 2003; Nichols and Smith, 2004). DNA extraction from environmental samples may contain organic compounds and humic substances that can inhibit qPCR reaction (Braid et al., 2003; Schriewer et al., 2011). The inhibitors may be removed by purifying the samples before or after DNA extraction, or during qPCR reaction (Jiang et al., 2005; Schriewer et al., 2011). Surfactants (e.g. Tween), multivalent cations (e.g. magnesium chloride, $MgCl_2$) and proteins (e.g. Bovine Serum Albumin, BSA) are used in qPCR mixture as inhibitor removal agents or PCR facilitators (Kreader, 1996, Jiang et al., 2005; Schriewer et al., 2011).

Detection and quantification of *C. parvum* oocysts in natural environments, such as soils, are critical to understand the fate and transport of oocysts, assess the risk of contamination of groundwater resources and potential threat to public health. Our research focuses on developing a qPCR protocol for the detection and quantification of *C. parvum* oocysts in natural soil matrices and leachates. Two different ages—1 month old (young) and 16 months old (aged)—of *C. parvum* oocysts were used in this study. Foremost, the physicochemical parameters—lysis

media (TE buffer, Chelex-100), number of thermal shocks (5, 10 or 15) and thawing temperatures (37, 65 or 95^{o}C)—controlling the DNA extraction efficiency were investigated and optimized to achieve the highest DNA yield for *C. parvum* oocysts. The optimum combination and concentration of PCR facilitators—$MgCl_2$ (1-4 mM) and BSA (200-600 ng/µl)—were examined to maximize the amplicon yield. The known numbers of *C. parvum* oocysts were disrupted and assayed under optimized conditions, and standard curves were generated to quantify the number of oocysts in soil and leachate samples. Latter, generated standard curves were used to quantify the number of *C. parvum* oocysts in soil and leachate samples. Volume of 40 ml DI water and Sparta, Greenson and Trenton soil leachate samples comprising final oocyst concentrations from 5×10^{1} to 5×10^{4} oocysts/ml were quantified. Two isolation methods—sucrose or two-phase flotation—were examined on 20 g soil samples from agricultural loamy sand (Gilford and Sparta), sandy loam (Lewiston), loam (Greenson) and silty loam (Trenton) soils comprising 10^{5} oocysts/g were quantified. Sucrose isolation method was further tested on 20 g soil samples from three different soils—Sparta, Greenson and Trenton—comprising final oocyst concentrations ranging from 10^{2} to 10^{5} oocysts/g were tested. The performance quantification was evaluated on the basis of PR values.

2. MATERIALS AND METHODS

2.1. *C. parvum* oocysts

Bovine-derived *C. parvum* oocyst (Iowa isolate) suspensions (Cat#P102C@1×10/9) were purchased from Waterborne Inc. (New Orleans, LA). Live *C. parvum* oocysts passed through experimentally infected calves and were extracted from fecal material using diethyl ether and purified by sucrose and Percoll™ density gradient centrifugation. A number of 1×10^9 *C. parvum* oocysts was quantified using Neubauer hemocytometer and suspended in 50 ml DI water with penicillin, streptomycin, gentamicin and amphotericin B. *C. parvum* oocysts of two different ages—1 month old (young) and 16 months old (aged)—were used in this study, and both oocysts suspensions were kept at 4-8^{o}C. Prior to each experiment, the supplied stock suspension of oocysts was mixed in a vortex mixer at 3,000 rpm for 15 min to disturb the pelleted *C. parvum* oocysts and to prepare homogenous suspensions.

2.2. Soils

Soil samples—loamy sand (Gilford and Sparta), sandy loam (Lewiston), loam (Greenson) and silty loam (Trenton) soils—were collected from permanently cultivated farmlands in the States of Illinois and Utah. The physical-chemical properties of soil were determined by the Soil Testing Laboratory at Utah State University (Table 1). ASTM 20/30 sand (Cat#C778, U.S. Silica Company, IL, USA) was also used as a soil for comparison.

TABLE I
PHYSICAL-CHEMICAL PROPERTIES OF SOIL SAMPLES

Soil Series	Trenton	Greenson	Lewiston	Sparta	Gilford
USDA Texture	Silty Loam	Loam	Sandy Loam	Loamy Sand	Loamy Sand
Sand (%)	18.5	66.9	79.7	82	84.4
Silt (%)	56.5	13.6	7.7	8.4	7.7
Clay (%)	25	19.5	12.5	9.5	7.9
Walkey-Black Organic Matter (%)	3.7	2.8	-	2.8	3.3
Loss of Ignition Organic Matter (%)	5.1	3.8	-	3.4	4.2
Total Carbon (%)	2.4	1.5	1.28	1.93	2.91
pH	8.5	7.4	-	6.9	5.2
Electrical Conductivity (dS/m)	1.8	0.7	-	0.36	0.68
Sodium Adsorption Ratio	15.1	1	-	-	-
Cation Exchange Capacity (meq/100g)	31	17.5	-	8.6	8.4

2.3. Sample preparation

2.3.1. Oocyst disruption and DNA extraction samples

Triplicate samples—each containing 2×10^6 *C. parvum* oocysts—were prepared to assess the effects of lysis media and physical disruption treatments—number of thermal shocks and thawing temperature— on DNA extraction from *C. parvum* oocysts. To prepare a sample, 100 µl *C. parvum* oocysts stock suspension was placed in a microcentrifuge tube and pelleted at 14,000×g for 5 minutes in a microcentrifuge (Model# 5424R, Eppendorf AG, Germany). Then, the supernatant was discarded and the pellets were washed twice and resuspended in a 300 µl lysis solution. Latter prepared samples were subjected to DNA extraction.

2.3.2. Water and leachate samples

DI water and three different leachate samples from Sparta, Trenton and Greenson soils were prepared to assess the performance of qPCR when applied to environmental waters. To prepare the leachate samples, an amount of 250 g of soil was added to 750 ml water and mixed using a magnetic stirrer for 15 minutes at 900 rpm. The suspension was left to settle for 15 minutes. The liquid phase was then transferred into 250 ml centrifuge bottles and centrifuged for 10 min at 1500×g. The main physical-chemical properties of leachates are presented in Table 2. Triplicate samples—each prepared with 40 ml supernatant—were spiked with 2×10^3, 2×10^4, 2×10^5 and 2×10^6 oocysts, resulting in oocysts concentration ranging from 5×10^1 to 5×10^4 oocysts/ml. The inoculated samples were mixed in vortex mixer at 2,000 rpm for 1 min and incubated for 24 hr at room temperature (24±2°C). Latter prepared samples were subjected to *C. parvum* oocysts concentration.

TABLE II
PHYSICAL-CHEMICAL PROPERTIES OF LEACHATE SAMPLES

Parameter	Unit	Trenton	Greenson	Sparta	DI Water
Conductivity	µS/cm	320.7	119.8	73.7	3.3
TDS	ppm	193.3	63.8	44.2	2.05
Salinity	ppt	0.155	0.050	0.033	0.001
Resistivity	kΩ	3.63	9.650	13.570	295
pH		9.55	7.15	7.01	6.70

2.3.3. Soil samples

Soil samples of 20 g were prepared in triplicates for testing the performance of *C. parvum* isolation methods. Two isolation methods—sucrose and two-phase flotation—were first tested on ASTM 20/30 sand, Gilford, Sparta, Lewiston, Greenson, and Trenton soil samples containing 10^5 *C. parvum* oocysts/g. The sucrose flotation method was further tested on Sparta, Greenson and Trenton soils to assess the effect of different inoculation concentrations (10^2, 10^3, 10^4 and 10^5 *C. parvum* oocysts/g) on the percentage of recovery. To prepare a soil sample, 20 g of soil was placed in a 250 ml conical bottom centrifuge tube (Cat#430776, Corning Inc, Corning, NY). Soil samples were inoculated with the required amount of *C. parvum* oocysts and saturated to 20% moisture content by adding 5 ml of DI water. Prior to inoculation, 100 µl *C. parvum* oocyst suspension was washed twice and suspended in 100 µl DI water to remove the manufacturer additives. The inoculated samples were mixed in vortex mixer at 2,000 rpm for 1 min and incubated for 24 hr at room temperature. Latter samples were subjected to *C. parvum* oocysts isolation.

2.4. Concentration of *C. parvum* oocysts in water samples

Environmental water samples—Sparta, Trenton and Greenson soil leachates— and DI water samples were centrifuged at 14,000×g in a Sorvall RC-5B centrifuge (DuPont Instruments, Newtown, CT) using SS-34 fixed angle rotor for 10 minutes. The supernatant (~ 38 ml) was discarded and the ~ 2 ml concentrate was transferred into a microcentrifuge tube and pelleted at 14,000×g for 5 minutes. The pellets were washed twice and resuspended in 300 µl lysis solution. Latter samples were subjected to DNA extraction.

2.5. Isolation of *C. parvum* oocysts from soil samples

A volume of 100 ml extraction solution containing 50 mM Tris (Cat#BP152, Fisher Scientific) and 0.5% Tween 80 (Cat#P8074, Sigma Aldrich, St.Louis, MO) was added to incubated soil sample (Mawdsley et al., 1996; Kuczynska and Shelton, 1999). Samples were placed in an orbital platform shaker and mixed gently at 175 rpm for 20 min. The suspension was subjected to two isolation methods—sucrose flotation and two-phase flotation. In the sucrose flotation method (Figure 1), the suspension was underlaid with 25 ml of cold sucrose (Cat#S2, Fisher Scientific) at concentration of 1.18 g/ml and centrifuged at 1,500×g in a swing-bucket rotor centrifuge for 15 min at 4^{o}C (Mawdsley et al., 1996). Whereas, in the two-phase flotation method, the suspension was underlaid by layers of 10 ml of 60% (w/v) PEG 8000 (Cat#BP233, Fisher Scientific) and 17 ml of 25% (w/v) sucrose dissolved in phosphate buffer (3 M, pH=7.1); then centrifuged at 500×g in a swing-bucket rotor centrifuge for 10 min at 4^{o}C (Zilberman et al., 2009). After density-gradient centrifugation, the upper ~75ml liquid layer was discarded and the lower 50 ml transferred into a 50 ml centrifuge tube. The suspension was washed with DI water

and centrifuged at 14,000×g in a fixed angle rotor centrifuge 5 min at 4^{o}C. Then the pellets were transferred into a microcentrifuge tube, pelleted at 14,000×g for 5 minutes in a microcentrifuge, washed twice and resuspended in 300 µl lysis solutions. Latter samples were subjected to DNA extraction.

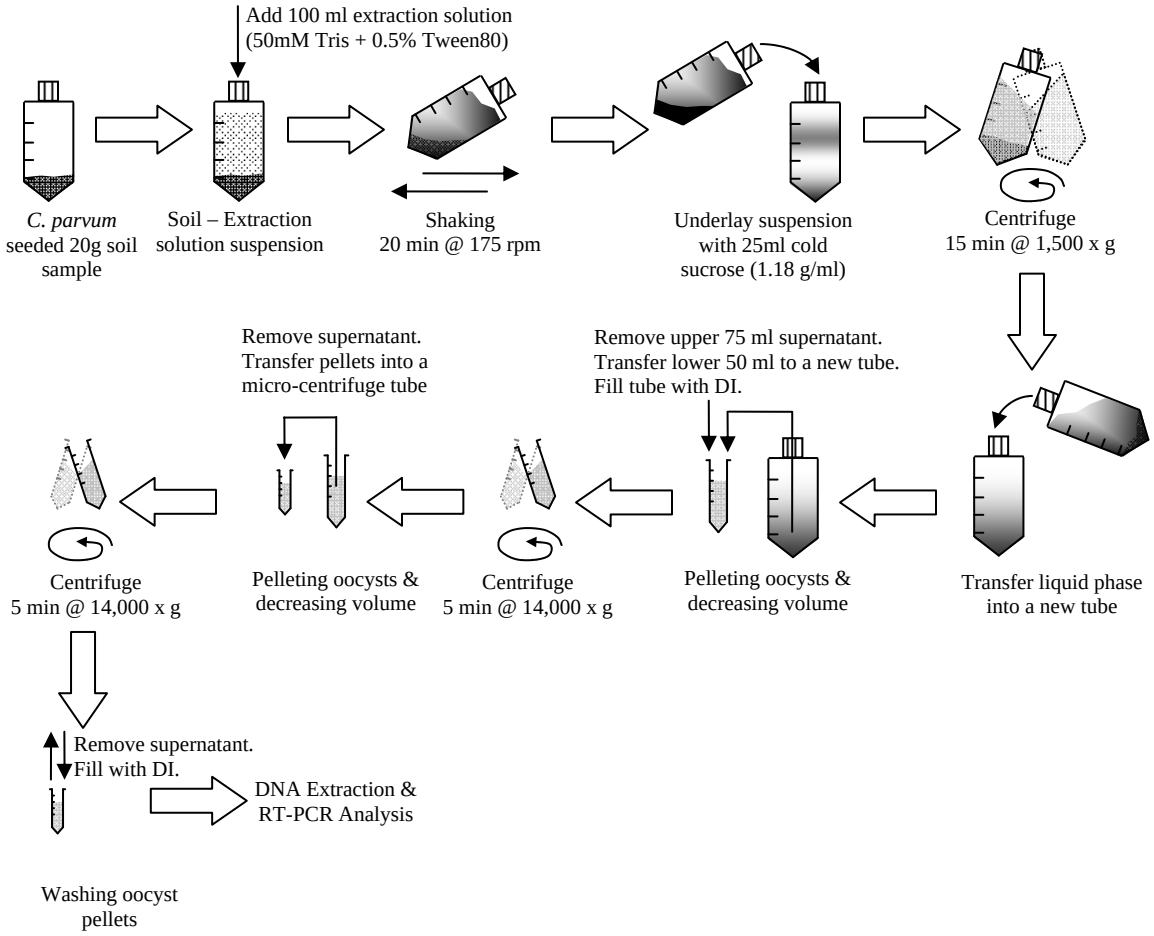

Figure 1. The process flow chart of *C. parvum* oocyst isolation from soil samples by sucrose flotation method.

2.6. DNA extraction

2.6.1. DNA extraction in Chelex-100

The genomic DNA of *C. parvum* was released in 300 µl 25% (w/v) Chelex-100 (Cat#143-2832, Bio-Rad, Hercules, CA) suspension by applying either 5, 10 or 15 cycles of thermal shocks—freezing at -80^{o}C in a dry ice-ethanol bath and thawing at either 37, 65 or 95^{o}C in an hot water bath. The duration of each hot/cold bath was 2 min (Fontaine and Guillot, 2002). At the end of thermal shocks, lysates were chilled on ice for 1 min. The debris was pelleted by centrifuging the lysate for 3 min at 14,000×g. The lysate was then subjected to DNA purification.

2.6.2. DNA extraction in Tris-EDTA (TE) buffer

Lysis buffer was prepared with 50 mM Tris-HCl (Cat#BP152, Fisher Scientific) at pH=8.5, 1 mM Ethylenediaminetetraacetic acid (EDTA) at pH=8.0 (Cat#H1758, Sigma Aldrich), and 0.5% (w/v) Sodium Dodecyl Sulfate (SDS) (Cat#71725, Sigma Aldrich) (Nichols and Smith, 2004). The DNA of *C. parvum* oocysts was released in 300 µl of TE buffer following the same thermal shock procedures as described in the case of DNA extraction in Chelex-100 (Figure 2). Following the thermal shocks, Proteinase K (Cat#EO0491, Fermentas Inc., Glen Burnie, MD) was added to the lysates at a final concentration of 200 µg/ml and incubated in a 55^{o}C water bath for 3 hours; thereafter denaturated by incubating the lysates in a 90^{o}C hot water bath for 20 min (Nichols and Smith, 2004). Lysates were chilled on ice for 1 min and centrifuged at 14000×g for 5 min. The lysate was then subjected to DNA purification.

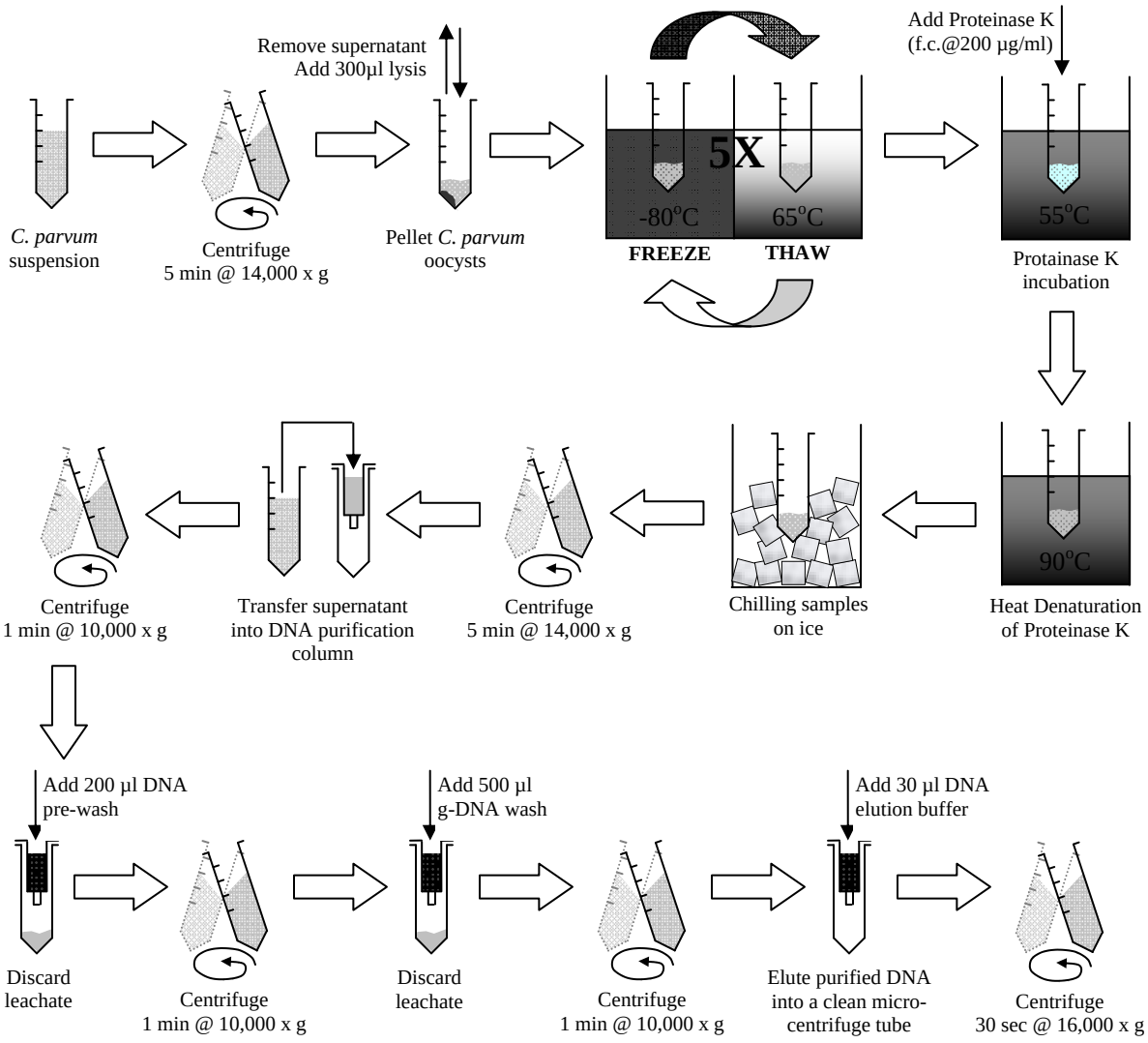

Figure 2. DNA extraction and purification process flow chart.

2.6.3. DNA purification

The lysate was pipetted onto a Zymo-Spin™ II DNA purification column (Cat#C1008, Zymo Research, Irvine, CA) and centrifuged at 10,000×g for 1 min. A volume of 200 µl DNA pre-wash buffer (Cat#D3004-5, Zymo Research) was added to the spin column and centrifuged again at 10,000×g for 1 min. A volume of 500 µl g-DNA wash buffer (Cat#D3004-2, Zymo Research) was added to the spin column and centrifuged 10,000×g for 1 min. The spin column was placed in a microcentrifuge tube and 30 µl of DNA elution buffer (Cat#D3004-4, Zymo Research) was pipetted directly on the silica of the spin column and incubated at room temperature for 5 min and centrifuged at 16,000×g for 30 seconds to elute the DNA into the microcentrifuge tube. Purified DNA samples were stored at -20^{o}C. Purified DNA was then used for qPCR.

2.7. qPCR

qPCR assay was prepared on basis of TaqMan® technology. The primers and TaqMan® probe were designed on the basis of GenBank accession number AF190627 by Jothikumar et al. (2008) as follows: 5'-ACTTTTTGTTTGTTTTACGCCG-3' (forward primer), 5'-AATGTG GTAGTTGCGGTTGAA-3' (reverse primer) and 5'-FAM-ATTTATCTCTTCGTAGCGGCG-BHQ-3' (probe). The oligonucleotides were synthesized by Sigma Aldrich. PCR reactions were performed using an ABI ViiA7 real-time PCR system. This system requires a minimum of 10 µl of reaction volume. The PCR mixture was prepared with 5 µl 2×TaqMan® Gene Expression Master Mix (P/N# 4369016, ABI, Carlsbad, CA), 1 µl purified DNA, primers and probe at final concentration of 300 nM and 200 nM, respectively. PCR facilitators—1-4 mM $MgCl_2$, 200-600

ng/µl BSA or combinations of $MgCl_2$ and BSA— were added to the assay to improve the amplicon yield. qPCR amplifications were performed using the following conditions: 1 cycle at 50°C for 2 min followed by 1 cycle of TaqMan® activation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 1 min. The variations of fluorescence during the PCR reactions were plotted against the cycle number. Statistically significant signals were distinguished by the threshold baseline. The signal intensity that crosses the threshold baseline is called the threshold cycle (Ct). The Ct is used for estimating the initial DNA copy number.

3. RESULTS

3.1. Comparisons of *C. parvum* oocyst disruption and DNA extraction method efficiencies

The physicochemical parameters controlling the DNA extraction efficiency were investigated to achieve the highest DNA yield for *C. parvum* oocysts. Triplicate samples—each containing 2×10^6 *C. parvum* oocysts—were disrupted in either TE buffer or Chelex-100 suspension by applying either 5, 10 or 15 cycles of thermal shocks thawing at either 37, 65 or 95^{o}C and assayed without any facilitators. The efficiency of oocyst disruption method was assessed by comparing the threshold cycle (Ct) values of amplified DNA of *C. parvum* oocysts (Table 3). The age of oocysts was determined to be the critical parameter affecting the oocyst disruption efficiency, as the young oocysts yielded significantly lower Ct values than the aged oocysts. Ct values ranged from 24.56 to 28.41 for young oocysts and from 27.30 to 37.06 for aged oocysts. The oocyst disruption efficiency was also impacted by the lysis media. In Chelex-100, the lowest Ct values were observed for young oocysts, while in TE buffer the lowest Ct values were detected for aged oocysts. The oocyst disruption was less dependent of the oocyst age in TE buffer than in Chelex-100. The difference between disrupting young and aged oocysts was about 1-2 cycles in TE buffer and 5-10 cycles in Chelex-100. The efficiency of the oocyst disruption was also impacted by thawing temperature and number of thermal shocks. In general, increasing the number of thermal shocks beyond five did not improve the disruption of oocysts.

The most efficient oocyst disruption method for C. parvum oocysts regardless of their age was then established as 5 thermal shocks with thawing at 65oC in TE buffer, producing Ct values of 26.48 and 27.30 for young and aged oocysts, respectively.

TABLE III

Ct VALUES OF YOUNG AND AGED *C. parvum* OOCYSTS DISRUPTED IN DIFFERENT LYSIS MEDIA, NUMBER OF THERMAL SHOCKS AND THAWING TEMPERATURES

Number of Thermal Shocks	Thawing Temperature (^{o}C)	Young *C.parvum*		Aged *C.parvum*	
		Chelex-100	TE Buffer	Chelex-100	TE Buffer
5	37	25.36 ± 0.43	26.21 ± 0.31	32.43 ± 1.04	28.73 ± 0.97
	65	24.86 ± 0.24	26.48 ± 0.20	30.15 ± 2.32	27.30 ± 0.40
	95	24.56 ± 0.43	26.93 ± 1.05	31.31 ± 1.22	27.55 ± 0.55
10	37	26.20 ± 1.02	26.18 ± 0.45	31.17 ± 1.34	28.38 ± 0.35
	65	25.15 ± 0.53	26.47 ± 0.40	31.45 ± 1.46	29.21 ± 0.64
	95	25.29 ± 0.38	28.41 ± 1.39	35.42 ± 1.30	28.51 ± 0.85
15	37	26.78 ± 0.46	26.71 ± 0.17	32.45 ± 1.39	28.14 ± 1.53
	65	24.87 ± 0.49	26.78 ± 0.50	30.07 ± 2.87	28.04 ± 1.12
	95	25.43 ± 0.24	28.33 ± 0.49	37.06 ± 1.25	27.83 ± 0.46

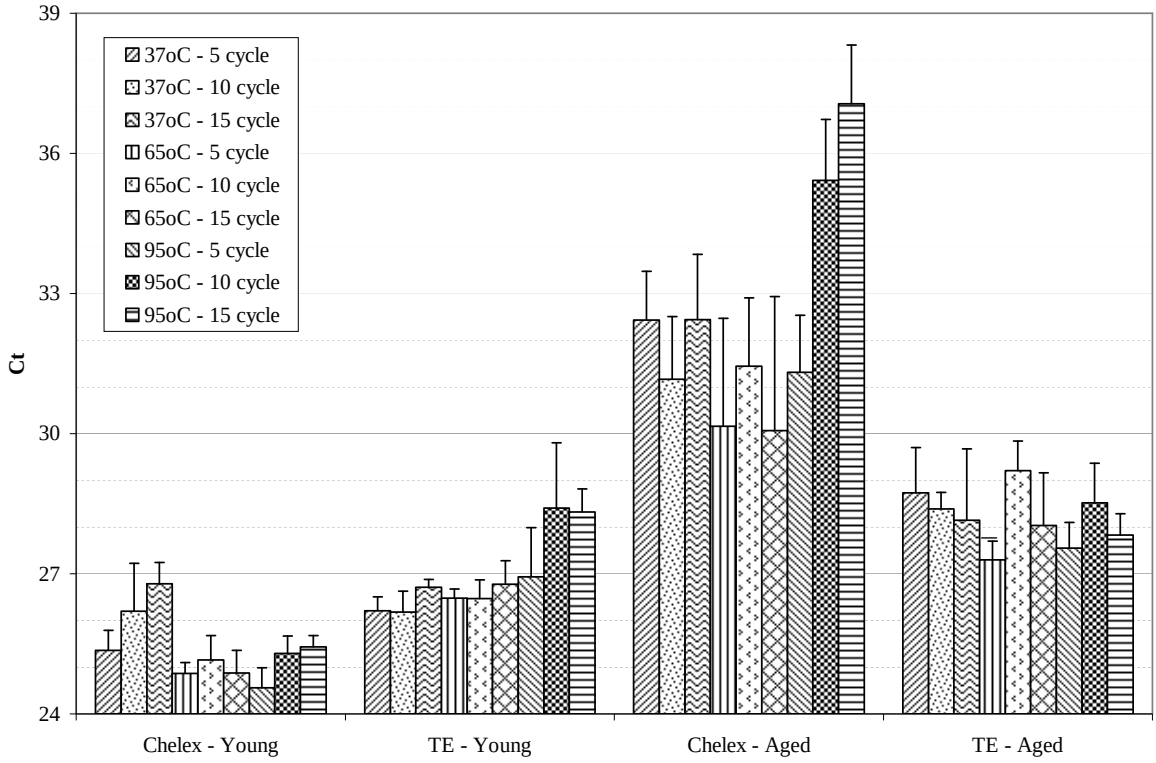

Figure 3. Graphical demonstration of Ct values of young and aged *C. parvum* oocysts disrupted in different lysis media, number of thermal shocks and thawing temperatures.

3.2. Effects of PCR facilitators

The optimum combination and concentration of PCR facilitators were examined to maximize the amplicon yield. Samples—each containing 2×10^6 *C. parvum* oocysts—were disrupted in TE buffer by 5 cycles of thermal shocks at thawing temperature of 65^{o}C and assayed in the presence of PCR facilitators—1-4 mM $MgCl_2$, 200-600 ng/µl BSA or various combinations of $MgCl_2$ and BSA (Table 4, Figure 4). The improvement in amplicon yield was assessed by comparing changes in Ct values resulting from addition of facilitators. The addition of facilitators were effective in improving the amplicon yield as the Ct values were lowered for both young and aged *C. parvum* oocysts, but the most notable improvements were observed in the case of aged *C. parvum* oocysts. The inclusion of any amount of either $MgCl_2$ or BSA lowered the Ct values by ~2-3 cycles for aged *C. parvum* oocysts. However, the combination of facilitators—3 mM $MgCl_2$ and 600 ng/µl BSA—improved the amplicon yield for both young and aged *C. parvum* oocysts. This combination led to Ct values of 25.24 for young oocysts, corresponding to a Ct value being lowered by more than 1 cycle, and 22.64 for aged oocysts corresponding to a Ct value being lowered almost 5 cycles (Figure 4). The facilitator combination was further tested on aged *C. parvum* DNA isolated from various soil types to observe the enhancement in amplicon yield for DNA extracted from environmental samples (Table 5, Figure 5). The facilitator combination was found less effective in environmental samples than clean samples and Ct values were lowered 1-2 cycles.

TABLE IV

Ct VALUES OF YOUNG AND AGED *C. parvum* OOCYSTS g-DNA AMPLIFIED IN THE PRESENCE OF DIFFERENT FACILITATOR COMBINATIONS AND CONCENTRATIONS

Facilitators	Young *C. parvum*	Aged *C. parvum*
without facilitators	26.48 ± 0.20	27.30 ± 0.40
1mM $MgCl_2$	27.02 ± 0.04	24.99 ± 0.04
2mM $MgCl_2$	26.68 ± 0.02	24.66 ± 0.12
3mM $MgCl_2$	26.33 ± 0.11	24.35 ± 0.04
4mM $MgCl_2$	26.12 ± 0.19	24.18 ± 0.07
200 ng/µl BSA	27.10 ± 0.05	24.97 ± 0.01
400 ng/µl BSA	26.89 ± 0.14	24.96 ± 0.02
600 ng/µl BSA	26.89 ± 0.07	24.91 ± 0.04
1mM $MgCl_2$ + 200 ng/µl BSA	26.86 ± 0.06	Undetermined
1mM $MgCl_2$ + 400 ng/µl BSA	26.81 ± 0.06	24.64 ± 0.07
1mM $MgCl_2$ + 600 ng/µl BSA	25.94 ± 0.67	24.08 ± 0.32
2mM $MgCl_2$ + 200 ng/µl BSA	26.55 ± 0.07	Undetermined
2mM $MgCl_2$ + 400 ng/µl BSA	26.43 ± 0.05	24.42 ± 0.01
2mM $MgCl_2$ + 600 ng/µl BSA	25.70 ± 0.55	23.63 ± 0.40
3mM $MgCl_2$ + 200 ng/µl BSA	26.14 ± 0.29	Undetermined
3mM $MgCl_2$ + 400 ng/µl BSA	25.75 ± 0.68	24.19 ± 0.07
3mM $MgCl_2$ + 600 ng/µl BSA	25.24 ± 0.71	22.64 ± 0.74
4mM $MgCl_2$ + 200 ng/µl BSA	26.07 ± 0.04	Undetermined
4mM $MgCl_2$ + 400 ng/µl BSA	25.89 ± 0.11	24.07 ± 0.02
4mM $MgCl_2$ + 600 ng/µl BSA	Undetermined	23.04 ± 0.90

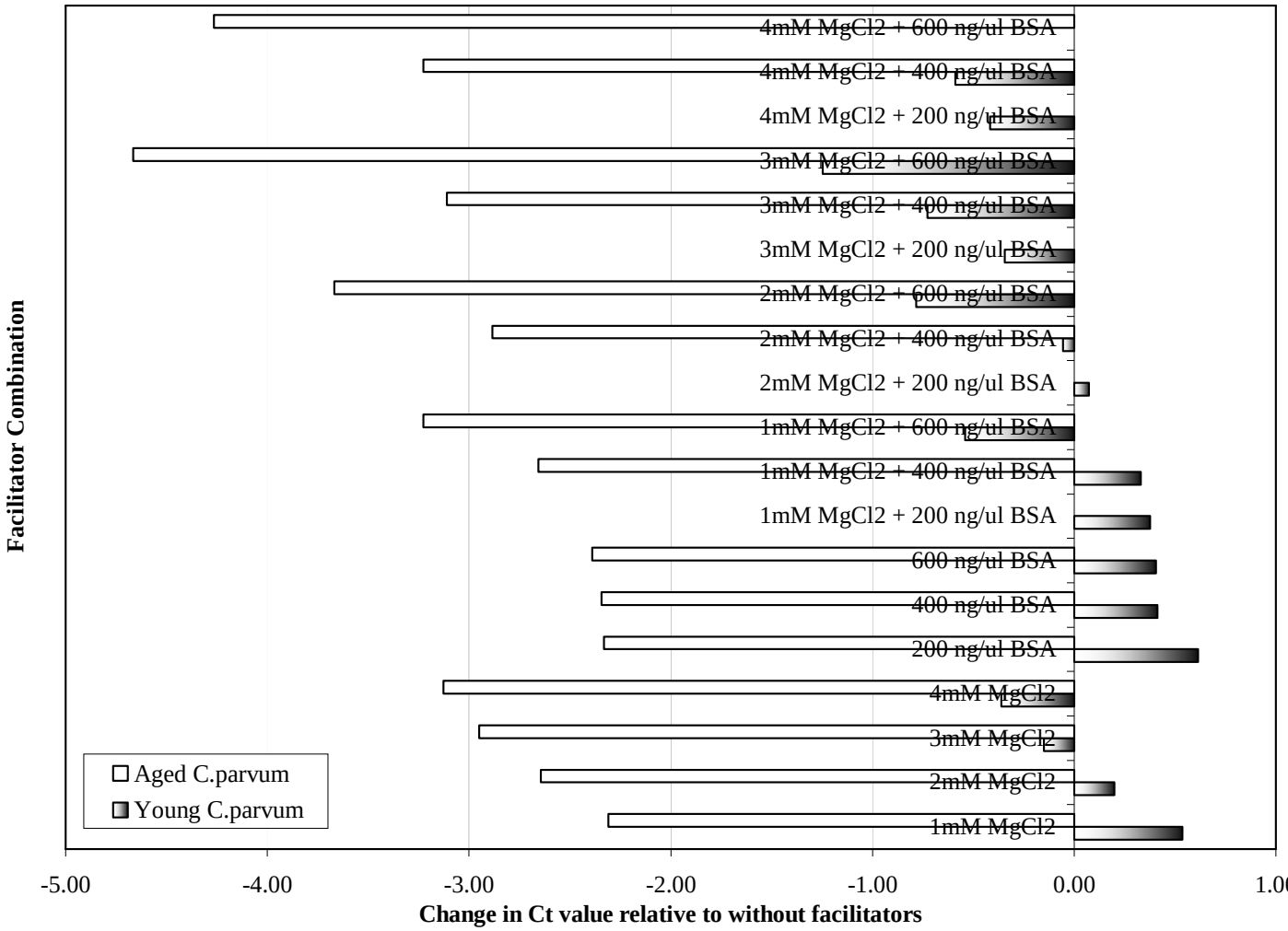

Figure 4. Relative changes in Ct values of young and aged *C. parvum* oocysts g-DNA amplified in the presence of different facilitator combinations and concentrations.

TABLE V

Ct VALUES OF AGED *C. parvum* OOCYSTS g-DNA ISOLATED FROM VARIOUS SOIL SAMPLES AND AMPLIFIED WITH AND WITHOUT 3mM $MgCl_2$ AND 600 ng/µl BSA

Soil Type	Soil Isolation Method	Amplified with facilitator combination	Amplified without facilitator combination
Trenton	Sucrose Flotation	35.238 ± 0.770	36.741 ± 0.545
Greenson		31.887 ± 2.058	33.194 ± 1.956
Lewiston		33.733 ± 1.835	34.596 ± 1.494
Sparta		32.067 ± 4.084	33.747 ± 4.488
Gilford		32.339 ± 0.770	33.247 ± 1.068
20/30 Sand		32.386 ± 0.687	33.228 ± 0.694
Trenton	2-phase Flotation	33.583 ± 0.683	35.076 ± 0.752
Greenson		32.978 ± 0.505	33.881 ± 1.052
Lewiston		33.397 ± 1.399	34.051 ± 1.958
Sparta		35.910 ± 0.665	36.965 ± 0.173
Gilford		34.324 ± 2.384	34.475 ± 1.781
20/30 Sand		31.513 ± 0.587	32.457 ± 0.565

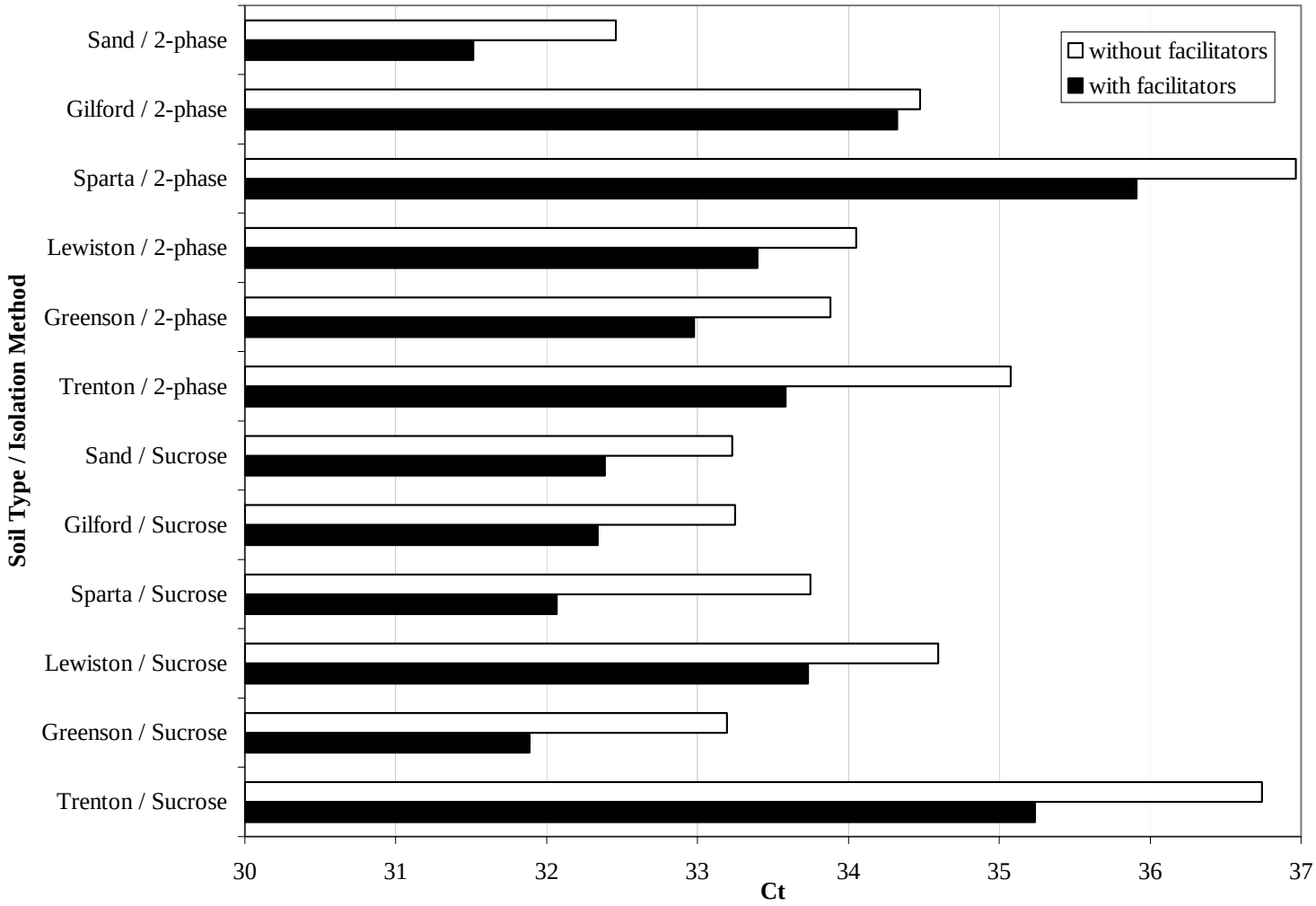

Figure 5. The effect of 3 mM $MgCl_2$ and 600 ng/µl BSA facilitator combination on amplification of aged *C. parvum* oocysts g-DNA isolated from various soil samples.

3.3. Standard curve

A standard curve was constructed to quantify the number of *C. parvum* oocysts in samples. Four replicates of reference samples—each including number of 6.6 x 10^4 *C. parvum* oocysts—were disrupted in TE buffer by 5 cycles of thermal shocks at thawing temperature of 65^{o}C. Serial 10-fold dilutions of DNA corresponding to number of oocysts ranging from 6.6 to 6.6×10^4 were prepared and four copies for each replicate dilution were amplified in the presence of 3 mM $MgCl_2$ and 600 ng/µl BSA. The Ct values (y-axis) were plotted versus log-concentrations of *C. parvum* oocysts (x-axis) to generate a standard curve (Figure 6). A linear regression was set to test the performance of the relationship, where y-intercept represented the theoretical limit of detection, the slope corresponded to the sensitivity of the serial dilutions, and the determination coefficient (r^2) reflected the accuracy of relationship (Fontaine and Guillot, 2003; Jothikumar et al., 2008). Linear regressions with 0.999 determination coefficients (r^2) were observed for both young and aged oocysts. The slope of standard curve was 3.386 for young oocysts and 3.556 for aged oocysts, comparing well with theoretical value of 3.333 and other experimental values ranging from 2.8 to 3.9 (Guy et al., 2003; Fontaine and Guillot, 2003; Jothikumar et al., 2008). The minimum detection limit was 0.667 for young and 6.67 (i.e. 200 oocysts) for aged *C. parvum* per PCR reaction (i.e. 2000 oocysts). A factor of 300 was used to convert the number of oocysts per PCR reaction to the numbers of oocysts in samples. This factor was obtained from dilution of DNA sample to PCR reaction.

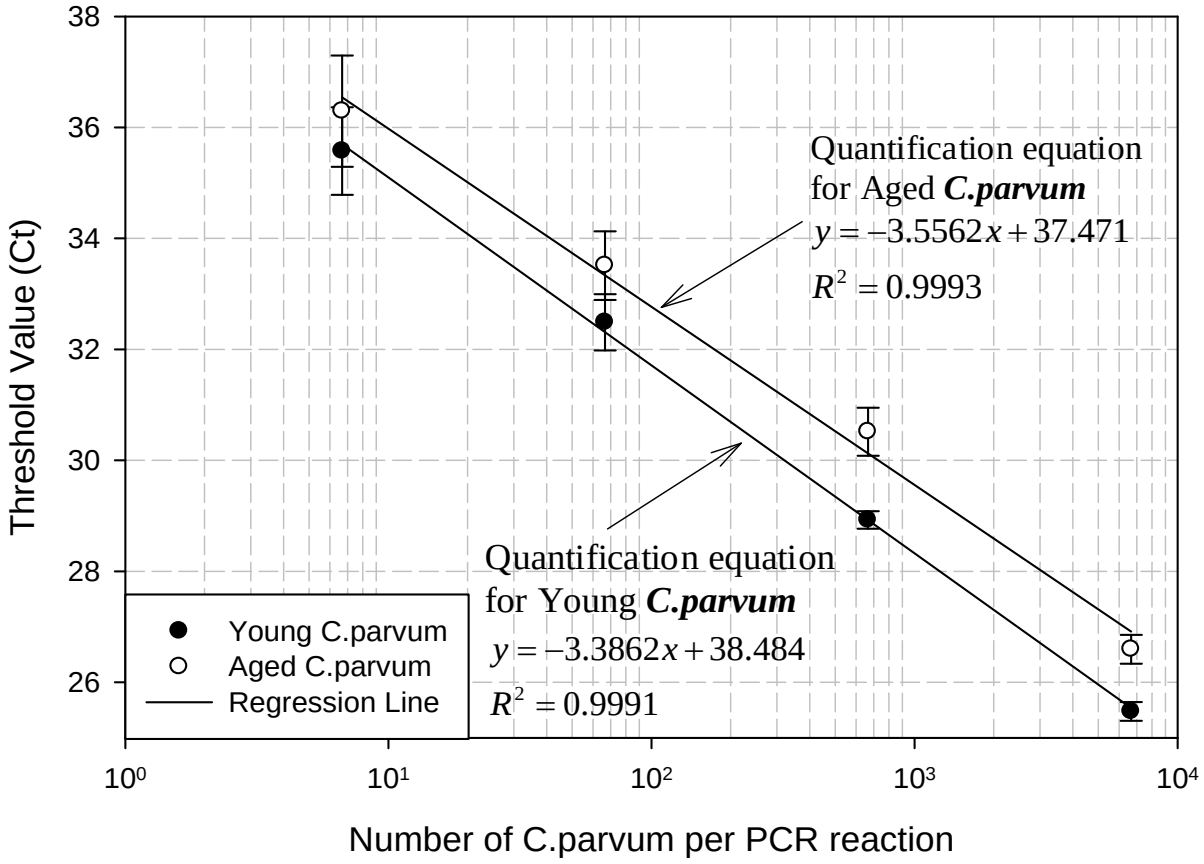

Figure 6. Linear regression between the number of *C. parvum* oocysts and corresponding Ct values (Standard curve).

3.4. Quantification of *C. parvum* oocysts in environmental water samples

DI water and Sparta, Greenson and Trenton soil leachate samples comprising final concentrations from 5×10^1 to 5×10^4 *C. parvum* oocysts/ml were quantified using the generated standard curves (Table 6, Figure 7). The PR values of *C. parvum* oocysts was determined as the ratio of the number of recovered *C. parvum* oocysts from samples and the number of *C. parvum* oocysts used to inoculate the samples. The average PR values for the ranges of oocyst concentrations used to inoculate the samples are 7.2-43.5%, 29.3-52.5%, and 11.5-60.8% for Trenton, Greenson, and Sparta soil leachates, respectively, and 12.1-77% for DI water.

TABLE VI

THE NUMBER OF INNOCULATED AND QUANTIFIED *C. parvum* OOCYSTS IN ENVIRONMENTAL WATER SAMPLES AND CORRESPODING PR VALUES

Soil	Innoculated Oocysts	Range	Ave ± St.Dev.	PR Range	Ave PR
Trenton	57867	16,975 - 24,497	21,367 ± 2,633	29.3 - 42.3	36.9
	4930	647 - 2,413	1,115 ± 485	13.1 - 48.9	22.6
	528	10 - 78	38 ± 21	2.0 - 14.7	7.2
	57	6 - 71	25 ± 27	11.0 - 125.2	43.5
Greenson	57867	19,863 - 44,201	30,403 ± 8,531	34.3 - 76.4	52.5
	4930	591 - 2,785	1,446 ± 627	12.0 - 56.5	29.3
	528	123 - 432	213 ± 93	23.2 - 81.9	40.4
	57	7 - 41	24 ± 13	12.6 - 72.2	41.4
Sparta	55628	22,933 - 40,858	31,905 ± 5,947	41.2 - 73.4	57.4
	4991	2,172 - 3,801	3,036 ± 601	43.5 - 76.2	60.8
	487	18 - 106	56 ± 23	3.7 - 21.8	11.5
	51	4 - 14	8 ± 4	8.4 - 27.1	16.1
DI Water	55628	27,609 - 36,763	31,577 ± 2,400	49.6 - 66.1	56.8
	4991	3,180 - 4,704	3,844 ± 549	63.7 - 94.2	77.0
	487	22 - 84	59 ± 18	4.4 - 17.3	12.1
	51	3 - 21	11 ± 6	6.1 - 42.1	22.1

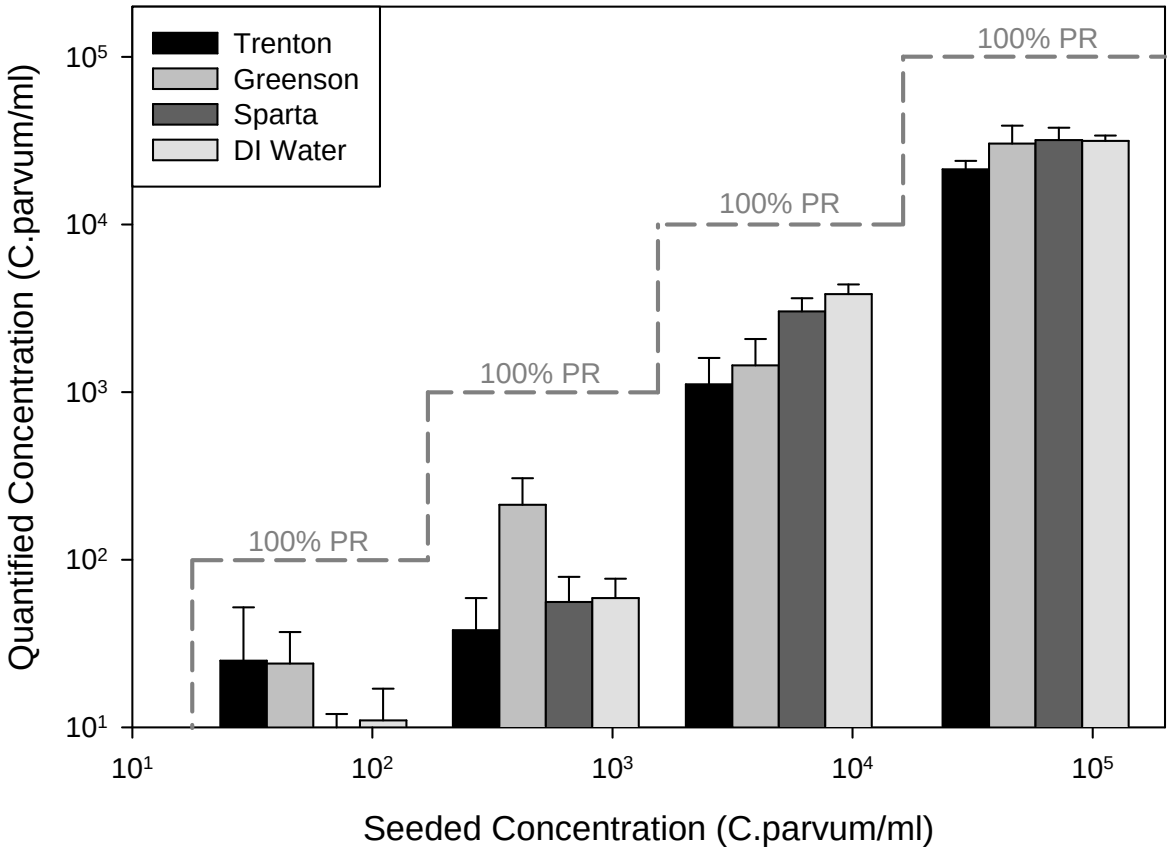

Figure 7. The number of quantified *C. parvum* oocysts in environmental water samples.

3.5. Isolations of *C. parvum* from Soil Samples

Two isolation methods—sucrose or two-phase flotation—were examined on contrasting soil types. Soil samples comprising 10^5 oocysts/g were quantified and PR values were calculated (Table 7 and Figure 8). The sucrose flotation method was found to be more efficient than the 2-phase flotation method to isolate young C. parvum oocysts from soil samples. The PR values of isolated young *C. parvum* oocysts by sucrose flotation are 2.6-9.6%, 2.2-.6.9%, 7.8-19.5%, 4.4-

15.9%, 4.2-10%, and 32.2-48.1%, for Gilford, Sparta, Lewiston, and Trenton soils, and 20/30 sand, respectively. The age of *C. parvum* oocysts has also an effect on both isolation methods. For each isolation method, 4 out of 6 PR values of isolated aged oocysts from soils were lower compared to the ones of young oocysts.

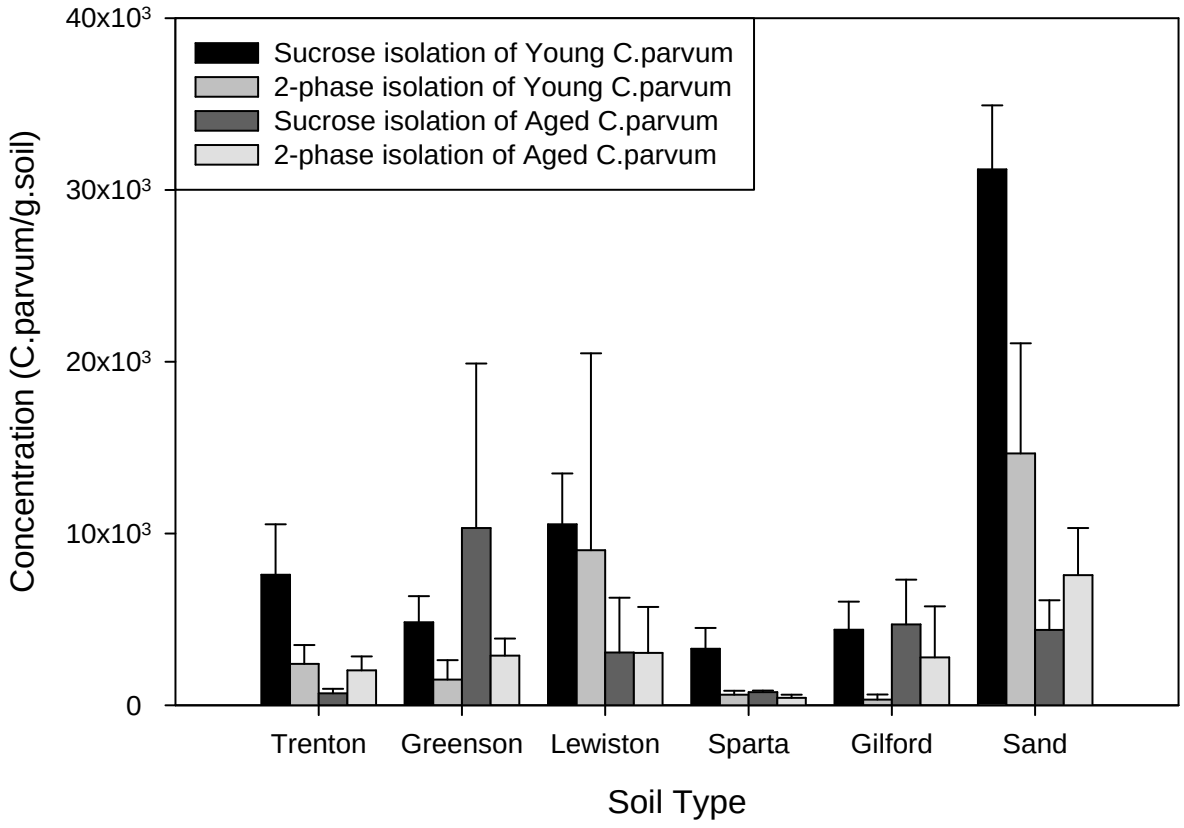

Figure 8. Performance of sucrose and two-phase flotation methods on isolation of young and aged *C. parvum* oocysts from various soil types.

TABLE VII

THE PR VALUES OF YOUNG AND AGED *C. parvum* OOCYSTS ISOLATED BY SUCROSE AND TWO-PHASE FLOTATION METHODS

Age	Method	Soil	Range	Ave ± St.Dev	PR Range	Ave PR
Young *C. parvum*	Sucrose Flotation	Trenton	3,334 - 12,054	7,591 ± 2,945	4.4 - 15.9	10.0
		Greenson	3,203 - 7,545	4,833 ± 1,510	4.2 - 10.0	6.4
		Lewiston	5,910 - 14,761	10,534 ± 2,952	7.8 - 19.5	13.9
		Sparta	1,633 - 5,201	3,281 ± 1,213	2.2 - 6.9	4.3
		Gilford	1,974 - 7,253	4,393 ± 1,637	2.6 - 9.6	5.8
		Sand	24,332 - 36,392	31,197 ± 3,721	32.2 - 48.1	41.2
Aged *C. parvum*	Sucrose Flotation	Trenton	726 - 4,088	2,407 ± 1,097	1.0 - 5.4	3.2
		Greenson	55 - 3,115	1,493 ± 1,135	0.1 - 4.1	2.0
		Lewiston	87 - 25,432	9,027 ± 11,458	0.1 - 33.6	11.9
		Sparta	140 - 938	607 ± 232	0.2 - 1.2	0.8
		Gilford	54 - 788	322 ± 301	0.1 - 1.0	0.4
		Sand	3,412 - 22,640	14,662 ± 6,412	4.5 - 29.9	19.4
Young *C. parvum*	Two Phase Flotation	Trenton	304 - 885	689 ± 264	0.4 - 1.2	0.9
		Greenson	585 - 24,994	10,310 ± 9,586	0.8 - 33.0	13.6
		Lewiston	281 - 8,049	3,069 ± 3,183	0.4 - 10.6	4.1
		Sparta	657 - 846	757 ± 95	0.9 - 1.1	1.0
		Gilford	2,367 - 9,838	4,702 ± 2,606	3.1 - 13.0	6.2
		Sand	1,912 - 7,495	4,385 ± 1,729	2.5 - 9.9	5.8
Aged *C. parvum*	Two-phase Flotation	Trenton	862 - 3,272	2,023 ± 814	1.1 - 4.3	2.7
		Greenson	1,524 - 5,318	2,891 ± 989	2.0 - 7.0	3.8
		Lewiston	715 - 7,544	3,048 ± 2,677	0.9 - 10.0	4.0
		Sparta	304 - 559	431 ± 180	0.4 - 0.7	0.6
		Gilford	274 - 7,025	2,784 ± 2,974	0.4 - 9.3	3.7
		Sand	3,560 - 13,166	7,571 ± 2,746	4.7 - 17.4	10.0

Sucrose isolation method was further tested on soil samples from three different soils—Sparta, Greenson and Trenton—comprising final oocyst concentrations ranging from 10^2 to 10^5 oocysts/g were quantified and PR values were calculated. The PR values of young *C. parvum* oocysts from Sparta, Greenson and Trenton soil samples are presented in Table 7 and Figure 6. As the *C. parvum* concentration decreased, the PR values increased with the highest (10.0-107.8%) and lowest (4.3-11.7%) PR values observed for Trenton and Sparta, respectively.

TABLE VIII

THE NUMBER OF INNOCULATED AND QUANTIFIED *C. parvum* OOCYSTS IN SOIL SAMPLES AND CORRESPODING PR VALUES

Soil	Innoculated Oocysts	Range	Ave ± St.Dev	PR Range	Average PR
Trenton	75655	3,334 - 12,054	7,591 ± 2,945	4.4 - 15.9	10.0
	7172	494 - 2,357	1,238 ± 601	6.9 - 32.9	17.3
	929	79 - 864	327 ± 305	8.5 - 93.0	35.2
	138	73 - 225	149 ± 107	52.7 - 162.9	107.8
Greenson	75655	3,203 - 7,545	4,833 ± 1,510	4.2 - 10.0	6.4
	7172	135 - 1,785	673 ± 626	1.9 - 24.9	9.4
	929	59 - 378	173 ± 115	6.4 - 40.7	18.6
	138	35 - 137	89 ± 45	25.7 - 99.3	64.2
Sparta	75655	1,633 - 5,201	3,281 ± 1,213	2.2 - 6.9	4.3
	7172	456 - 1,156	782 ± 183	6.4 - 16.1	10.9
	929	30 - 245	109 ± 70	3.2 - 26.3	11.7
	138	UD	UD	UD	UD

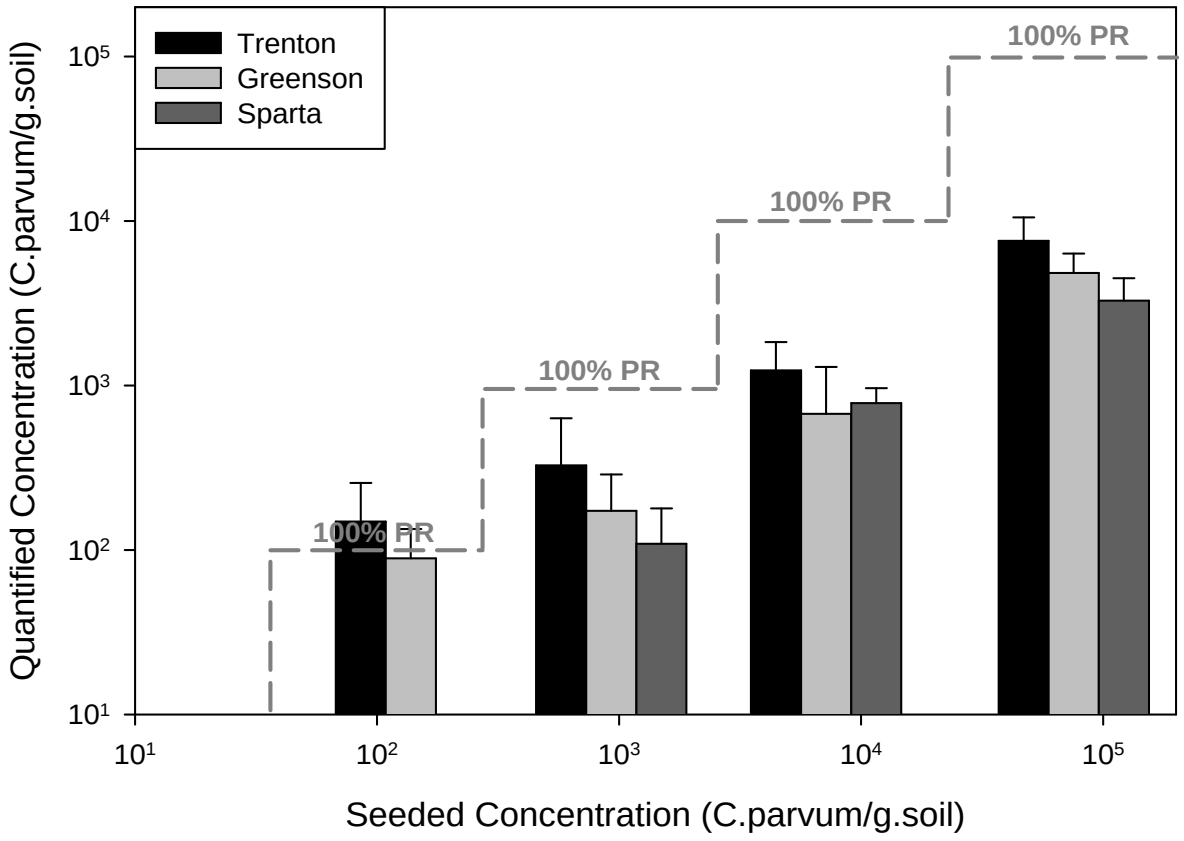

Figure 9. The number of quantified *C. parvum* oocysts in soil samples.

4. DISCUSSION

4.1. Optimization of C. parvum oocyst disruption and DNA extraction parameters

The optimization of oocyst disruption and DNA extraction parameters —lysis media, number of thermal shocks and thawing temperature— is critical in quantification of *C. parvum* using qPCR method. The oocyst age was found to be the most critical parameter affecting the oocyst disruption, as also reported by Monis (2003) and Nichols and Smith (2004). As described by Jenkins et al. (1997), Inoue et al. (2006) and Lalonde and Gajadhar (2009), less amplifiable DNA is available when oocysts are aging as related to their wall permeability; a decrease in DNA extraction sensitivity is observed after 15-months, even if the oocysts are stored in ideal conditions. The decrease in the DNA yield that we observed between 1- and 16-months old Iowa isolate *C. parvum* oocysts (Table 3, Figure 3) may be attributed to a lower amount of amplifiable DNA resulting from more permeable oocyst walls.

The impact of oocyst age on DNA extraction was found to be less dependent when oocysts were disrupted in TE media than in Chelex-100 buffer. While the difference between disrupting young and aged *C. parvum* was around 5-10 cycles in Chelex-100 buffer, it was as low as 1 cycle in TE buffer (Table 3, Figure 3). According to the microscopic investigation that Nichols and Smith (2004) performed, TE media assisted the release of DNA from disrupted oocysts and yielded higher DNA compared to PCR buffer and reverse osmosis water. Moreover the use of Proteinase K treatment following thermal shocks in TE media considerably improved

the DNA yield. Similarly, Monis et al. (2003) observed that oocysts treated with trypsin prior to thermal shocks decreased the effect of oocyst age on DNA extraction. Such enzymatic treatments have been applied to *C. parvum* oocysts to strip off the glycocalyx layer formed on the surface of oocyst (Harris and Petry, 1999; Dumetre et al., 2011). Thus, the digestion of glycocalyx formation might be the critical factor to the improvement of DNA extraction method in TE buffer, as it is impacting proteolysis and antibody binding (Nanduri et al., 1999).

Although the disruption of oocysts in TE buffer diminished the age effect, young oocysts disrupted in Chelex-100 yielded marginally lower Ct values more than oocysts disrupted in TE (Table 3, Figure 3) which was also reported by Hallier-Soulier and Guillot (1999) and Lowery et al. (2000). This would be attributed to the DNA degradation during thermal shocks in Chelex-100; which would possibly less than TE media. Chelex-100 resin is known to prevent DNA degradation during the thermal shocks (Hallier-Soulier and Guillot, 1999; Fontaine and Guillot, 2003).

Aged oocysts were more resistant to disruption by thermal shocks (Nichols and Smith, 2004); hence, changes in thawing temperature and number of thermal shocks were more remarkable in aged than young oocysts. While young oocysts thawed at either 37 or 65^{o}C in TE media yielded the lowest Ct values, 37^{o}C was inefficient to disrupt aged oocyst and resulted in over 2 cycles of increase in Ct values. Besides, the deficiency of thawing young oocysts in TE media at 95^{o}C for longer than 5 thermal shocks might be attributed to DNA degradation. Similarly, aged oocysts disrupted under the same conditions but in Chelex-100 media yielded

almost 5 cycles of higher Ct value compared to the average; and may be attributed to DNA degradation.

We determined TE buffer to be a better lysis media for DNA extraction considering help of TE buffer releasing DNA from disrupted oocysts, reducing the importance of oocysts age as oocysts disrupted in TE buffer, easiness of preparation, application and cost of the buffer. Thawing temperature 65^{o}C and five cycles of thermal shocks were the most efficient for both young and aged oocysts considering the Ct value and its standard deviation. Thus, the most efficient oocyst disruption method for *C. parvum* oocysts regardless of their age was then established as 5 thermal shocks with thawing at 65^{o}C in TE buffer.

4.2. DNA quality and amplification improvement

The oocyst disruption method was coupled with purification columns to remove PCR inhibitors such as SDS, EDTA and Proteinase K that were used to prepare the lysis (TE) media and environmental impurities such as complex polysaccharides, humic substances that may present in environmental water and soil samples (Kreader, 1995; Rådström, et al., 2004). Besides, PCR facilitators were tested to remove the residual inhibitors and improve the amplicon yield. We demonstrated that Ct values were affected not only by facilitator combination and concentration but also oocyst age. While the additional BSA increased Ct values almost half cycle for young *C. parvum* oocysts, it decreased Ct values more than 2 cycles for aged *C. parvum* oocysts. Guy et al. (2003) has also reported that additional 20 ng/µl BSA decreased the efficiency of amplification for 3-months-old *C. parvum* oocysts. Similarly additional $MgCl_2$

caused an increase in Ct values, but followed with a decrease as the concentration of $MgCl_2$ increased for young *C. parvum* oocysts. Inclusion of any amount of $MgCl_2$ was effective and yielded almost 3 cycles of decrease in Ct value for aged *C. parvum* oocysts. However, the combination of 3 mM $MgCl_2$ and 600 ng/µl BSA decreased Ct values by almost 5 cycles for aged and by more than 1 cycle for young *C. parvum* oocysts (Table 4, Figure 4). The facilitators were more necessary to enhance the assays for aged than young *C. parvum* oocysts. This might be again attributed to the degradation of DNA due to the degradation of oocyst wall, because the target of selected primer set was undefined. While additional $MgCl_2$ is suppressing the inhibitors targeting Taq polymerase as reported McCord et al. (2011), additional BSA is stabilizing inhibitors associated with extracted DNA (Kreader, 1996).

All environmental samples analyzed with DNA purification columns and without facilitators yielded positive PCR reactions (Table 5, Figure 5). This result revealed that DNA purification columns were effective to remove essential inhibitors and yielded PCR-quality DNA. However, additional 3 mM $MgCl_2$ and 600 ng/µl BSA improved the amplicon yield by decreasing Ct values almost 2 cycles. Many studies were in agreement with the utilization of BSA in PCR assays to remove the inhibitory effects of humic substances in environmental samples (Kreader, 1996; Guy et al., 2003; Jiang et al., 2005; Schriewer et al., 2011); however the required amount of BSA seemed to vary according to the utilized DNA extraction method, composition of PCR assay and amount of available inhibitors in environmental samples.

4.3. Specificity and sensitivity of detections and quantifications of C. parvum

Immunonological assays followed by microscopic examinations are commonly applied method in detection and quantification of *C. parvum* in environmental samples (Morgan and Thompson, 1998; Jex et al., 2008; Jothikumar et al., 2008). However most of these methods are lack of sensitivity and specificity (Morgan and Thompson, 1998; Jex et al., 2008). Besides, the reliability of examination is highly dependent on examiner's microbiological identification and microscopy skills (Morgan and Thompson, 1998; Jothikumar et al., 2008). qPCR is a sensitive and straightforward method which overcomes the limitations in traditional detection methods and enables direct quantification (Johnson et al., 1995).

The developed TaqMan assay provided very specific and sensitive detections. The sensitivity of the detections was on the grounds of employed *C. parvum* specific primer-probe set developed by Jothikumar et al. (2008). This primer-probe set was only cross-reacted with *C. wairi* among 10 analyzed *Cryptosporidium* species and it was more specific than most of the designed primer-probe sets in the literature (Johnson et al., 1995; Lowery et al., 2000; Fontaine and Guillot, 2002; Jothikumar et al., 2008). Although the primer-probe set that Guy et al. (2003) used in PCR assays was more specific than the one used in our study, it was targeting the oocyst-wall protein gene thus the performance of PCR detections might be more dependent to the oocyst age due to the degradation of oocyst wall as the oocysts get aged.

Although all measures were taken to diminish the oocyst age effect by optimizing oocyst disruption, improving DNA quality and employing additional facilitators to remove residual

inhibitors and enhance the amplicon yield, the gap between the Ct values of young and aged *C. parvum* oocysts shrunk but not at desired level. We therefore established two standard curves for quantification of young and aged *C. parvum* oocysts. The standard curves had both determination coefficients (R^2) of 99.9% which compared well with Jothikumar et al. (2008). The slopes of standard curves were found as 3.386 for young and 3.556 for aged *C. parvum*, which also compared well with theoretical value of 3.32 and other experimental values ranging between 2.8-3.9 (Guy et al., 2003; Fontaine and Guillot, 2003; Jothikumar et al. 2008). The y-intercept value was observed as 38.484 for young and 37.471 for aged *C. parvum* and both were within the analyze limit of 40 cycles; besides, significantly lower and more desirable than other studies (Guy et al., 2003; Fontaine and Guillot, 2003). The minimum detection limits were as low as 0.67 young and 6.7 aged *C. parvum* oocysts per PCR reaction and in agreement with findings from other researchers (Fontaine and Guillot, 2003; Guy et al., 2003; Jothikumar et al., 2008). The sensitive results might be attributed to sensitivity of pipeting during the preparation of 10-fold dilutions, the composition of PCR assays as well as the technology performance and calibration of thermocycler.

4.4. Concentration of environmental water samples

C. parvum oocysts were found at low concentrations in the environment and high sample volumes were required to detect and quantify the number of oocysts. However molecular biology methods were applicable at very low sample volumes. Therefore effective and easy concentration method was necessary to concentrate and prepare samples for DNA extraction. IMS was the most common applied method among the filtration, gradient flotation and IMS

methods; however compromising PR values were reported in the literature (Hsu and Huang, 2001; McCuin and Clancy, 2005; Carey et al., 2006). We achieved mean PR values of 40±20% Trenton, Greenson and Sparta soil leachates and 43±13% for DI water using high-speed centrifugation. The results that we achieved in this study were higher than most of the gradient flotation results and comparable with IMS results reported in the literature (Chesnot and Schwartzbrod, 2004; McCuin and Clancy, 2005; Carey et al., 2006). Besides, we found that high-speed centrifugation was independent to the sample turbidity in contrast to IMS method (Lowery et al., 2000; McCuin and Clancy, 2005). Unlike Carey et al. (2006), we could not establish substantive relationship between seeding concentrations and PR values. The high standard deviation in PR values might be attributed to the experimental errors during aspiration of supernatant, transferring pellets between the tubes and loss of oocysts during washing. Filtration-based methods would be more easy and less-dependent to experimental errors. Chesnot and Schwartzbrod (2004) reported highest PR values with lowest standard deviation for concentration of *C. parvum* oocysts in various water environments using filtration method. Therefore, filtration method might be the most efficient and economic solution for concentration of environmental water samples including raw, secondary and tertiary wastewater, river water and soil leachates.

4.5. Isolation of *C. parvum* oocysts from natural soil samples

Isolation method plays key role in quantification of *C. parvum* oocysts in natural soils. The performance of different methods –sucrose and two-phase flotation- were assessed using the PR values of *C. parvum* (Table 7). The PR values of isolated young *C. parvum* oocysts by

sucrose flotation are 2.6-9.6%, 2.2-.6.9%, 7.8-19.5%, 4.4-15.9%, 4.2-10%, and 32.2-48.1%, for Gilford, Sparta, Lewiston, and Trenton soils, and 20/30 sand, respectively. The achieved PR results were comparable with results available in literature (Mawdsley et al., 1996; Kuczynska and Shelton, 1999). However in our study, the PR values were obtained from seeding 20 g of soils compared to 1 g in the study of Mawdsley et al. (1996), and incubating samples for 24 hr of compared to 1 hr in the study of Kuczynska and Shelton (1999). Our result and parameters of our study such as volume of samples and time of incubations supported well the applications of our method to detection of *C. parvum* in the environment under natural conditions and reasonable sampling size and time procedures.

5. CONCLUSION

This research was focused on developing a qPCR protocol for sensitive and specific detection and quantification of *C. parvum* in natural soil matrices and leachates. The physicochemical parameters—lysis media (TE buffer, Chelex-100), number of thermal shocks (5, 10 or 15) and thawing temperatures (37, 65 or 95^{o}C)—controlling the DNA extraction efficiency were investigated. The oocyst age was found to be the most critical parameter affecting the oocyst disruption and it was found to be less dependent when oocysts were disrupted in TE than in Chelex-100 buffer. Changes in thawing temperature and number of thermal shocks were found more remarkable in aged than in young oocysts and increasing the number of thermal shocks beyond five did not improve the oocyst disruption. The most efficient oocyst disruption method for *C. parvum* oocysts regardless of their age was then established as 5 thermal shocks with thawing at 65^{o}C in TE buffer. The DNA extraction method was coupled with purification columns to remove PCR inhibitors in environmental samples and additional PCR facilitators—$MgCl_2$ and BSA—were examined to remove residual inhibitors and improve the amplicon yield. The combination of 3 mM $MgCl_2$ and 600 ng/µl BSA yielded highest amplicon yield for both young and aged oocysts. The optimized parameters of DNA extraction and qPCR assay provided very specific and sensitive detections of *C. parvum* in our study. The minimum detection limit was 0.667 for young and 6.67 for aged *C. parvum* per PCR reaction and the accuracy of the detections and quantifications were 0.999. The performance of developed protocol was further tested in contrasting soil and leachate samples on the basis of PR values. Mean PR values were calculated as 40±20% Trenton, Greenson and Sparta soil leachates and

43±13% for DI water. Sucrose flotation was determined as a better isolation method than two-phase flotation and it was further experimented for varying concentrations of *C. parvum.* The lowest and highest PR values were determined as 4.3% and 107.8%, respectively.

To our knowledge, this study is the first to apply qPCR for detection and quantification of *C. parvum* oocysts in natural soil matrices and leachates. This method can find application to environmental water quality, water management and agricultural management for the protection of public health.

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