Applicability of quantitative PCR for determination of removal efficacy of enteric viruses and *Cryptosporidium* by water treatment processes

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ABSTRACT

This study aims to assess the applicability of quantitative PCR (qPCR) for removal studies of adenovirus, coxsackievirus, echovirus and *Cryptosporidium* by water treatment processes. Bench-scale coagulation jar tests were performed using the enteric viruses and *Cryptosporidium*. Standard methods (conventional cell-culture methods for the viruses and an immunofluorescence assay (IFA) for *Cryptosporidium*) were used to compare to qPCR. A significant correlation between microbial removals determined by qPCR and the standard detection methods and an approximate 1:1 correlation were observed for the challenge microorganisms. The results indicated that qPCR could be a satisfactory alternate for microbial removal studies using a relative quantification approach.

Key words | *Cryptosporidium*, enteric viruses, quantitative PCR, water treatment processes

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INTRODUCTION

Multi-barrier drinking water treatment processes have demonstrated efficiency in the removal and inactivation of a variety of microorganisms, including viruses, bacteria and protozoa (Jofre *et al.* 1995; Gerba *et al.* 2003; Xagoraraki *et al.* 2004; Abbaszadegan *et al.* 2007, 2008). Accordingly, waterborne disease outbreaks associated with contaminated drinking water have decreased significantly in recent years in the United States (US) (Craun *et al.* 2006). However, community outbreaks of gastroenteritis still occur due to breakdowns and overloading of public water utilities with emerging or re-emerging pathogens. In an effort to provide higher quality drinking water to the public, the US Environmental Protection Agency (EPA) continually develops and re-evaluates regulations targeting drinking water contaminants, including the protozoan parasite *Cryptosporidium* (USEPA 2006). In addition, the USEPA periodically revises the drinking water Contaminant Candidate List (CCL), which is a list of unregulated contaminants warranting priority research (USEPA 2008). In order to evaluate the CCL for determination of future regulations, microbial treatability by water treatment processes must be considered.

To date, there are limited data on the physical removal of CCL viruses during water treatment processes (Gerba *et al.* 2003; Abbaszadegan *et al.* 2007, 2008; Mayer *et al.* 2008). One reason for this limitation is the practical difficulty of performing bench- or pilot-scale experiments for the removal studies of microbial pathogens, particularly in microbial detection methods. Relatively high numbers of samples are generally required for water treatment performance evaluation studies. Cell-culture-based infectivity assays have traditionally been used as a standard detection

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method for enteric viruses. It is difficult to handle multiple samples simultaneously because of the intensive labor requirements and time-consuming procedures for cell-culture assays. This suggests that the feasible number of samples which could be processed is very limited. Moreover, for some enteric viruses, such as coxsackievirus and echovirus, traditional cell-culture assays require separate experiments for each virus since they infect the same cells (e.g. buffalo green monkey kidney [BGMK] cells). Consequently, the number of samples is significantly increased.

An immunofluorescence assay (IFA) followed by epifluorescence microscopy has traditionally been used as a standard detection method for *Cryptosporidium* oocysts (USEPA 2005). However, epifluorescence microscopy is time consuming, and should not be performed for more than 4 hours per day nor more than 5 consecutive days per week for an analyst, thereby limiting the feasible number of samples (USEPA 1996). Furthermore, in slides prepared using environmental water samples, there is often a high level of background fluorescence, which makes it difficult to count oocysts and consequently increases microscopy time.

For the last decade, molecular techniques such as polymerase chain reaction (PCR) have been used intensively in a variety of applications. More recently, many researchers have used real-time PCR to detect and quantify a suite of microorganisms (Donaldson *et al.* 2002; Guy *et al.* 2003; Skovhus *et al.* 2004; He & Jiang 2005; Gregory *et al.* 2006). Real-time PCR is a promising technique that is both rapid and quantitative and can be used on various types of samples. The main objective of this study was to assess the applicability of quantitative real-time PCR (qPCR) for removal studies of adenovirus, coxsackievirus, echovirus and *Cryptosporidium* oocysts by water treatment processes in comparison to the standard detection methods. This study also includes the efficacy of the removal of oocysts under a range of enhanced ferric chloride coagulation conditions.

MATERIALS AND METHODS

Preparation of enteric viruses and *Cryptosporidium parvum* oocysts

Adenovirus type 4 (Ad4, ATCC VR-4), coxsackievirus B6 (CoxB6, ATCC VR-155) and echovirus type 12 (Echo12, ATCC VR-1563) were obtained from the ATCC. Ad4 was cultured using primary liver cancer cells (PLC/PRF/5, ATCC CRL-8024) in 1 × Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum (FBS). CoxB6 and Echo12 were cultured using buffalo green monkey kidney (BGMK) cells in 1 × MEM containing 5% FBS. The 1 × MEM was supplemented with 1.5 g/L sodium bicarbonate, 15 mM Hepes, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 100 μg/mL antimycotic and 100 μg/mL kanamycin sulfate. Following propagation of virus stocks, the virus suspensions were further purified and concentrated using polyethylene glycol (PEG) precipitation, as previously described by Abbaszadegan *et al.* (2007). To remove lipids and facilitate the monodispersion of virus clumps, a Vertrel® XF (Micro Care Marketing Services, New Britain, CT) extraction was performed by centrifuging a suspension of equal parts Vertrel and virus at 4°C at 8,000 × *g* for 90 min (Thurston-Enriquez *et al.* 2003). The supernatant containing the purified viruses was stored at − 80°C.

Freshly purified *Cryptosporidium parvum* oocysts (Iowa isolate) were obtained from the Sterling Parasitology Laboratory at the University of Arizona, Tucson, AZ, within 2 weeks after shedding. The number of oocysts in the stock suspensions was confirmed by direct count using a hemacytometer as described by the USEPA (2005). In order to calculate a mean spike dose, 10 aliquots of spiking suspension were enumerated.

Jar testing

Bench-scale jar tests using untreated central Arizona surface water were designed for enhanced coagulation with various coagulant doses and pH adjustment, as described by Abbaszadegan *et al.* (2007). Briefly, 10 L of water of turbidity 9 NTU, pH 8, alkalinity 140 mg/L as $CaCO_3$ and DOC 4 mg/L was seeded with a known number of three CCL viruses and *C. parvum* oocysts (final concentration of $10^{3.5}$ 50% tissue culture infectious dose [$TCID_{50}$]/mL of each virus and 1×10^3 oocysts/mL). The water was distributed to a Phipps & Bird PB-700 (Richmond, VA) jar test apparatus at a volume of 1.5 L per jar. The jars were treated using varying coagulant doses (from 0 to 120 mg/L

$FeCl_3$ with increments of 20 mg/L) and pH adjustment (down to 5.5 with decrements of 0.5).

Following coagulation and sedimentation, 10 mL samples were collected from the center of each jar, approximately 5 cm below the surface for virus analysis. A Vertrel® XF extraction was repeated to facilitate the monodispersion of virus clumps in the samples, followed by filter sterilization using a 0.22-μm-pore-size filter (Millipore Corp., Bedford, MA). The purified 10 mL samples were each divided into two aliquots for cell-culture analyses and qPCR. For *Cryptosporidium* analyses, 50 mL of water was collected from the center of each jar. The water samples were centrifuged at 1,800 × *g* for 10 min. The concentrates were suspended in 1.5 mL of 1 × phosphate buffer saline (PBS) and subsequently centrifuged at 20,000 × *g* for 2 min without braking. The supernatant was removed by aspiration, and the pellet was resuspended in 200 μL of 1 × PBS and then divided into two 100-μL aliquots for IFA and qPCR.

Standard detection methods for enteric viruses and *C. parvum* oocysts

Following jar test experiments, the water samples were serially diluted (10-fold) in 1X PBS (sufficient for up to 4.5-log removal) and were promptly assayed using conventional *in-vitro* cell-culture methodology: cytopathogenic effects (CPE) assay for Ad4 and plaque forming unit (PFU) assay for CoxB6 and Echo12. For the CPE assay, PLC/PRF/5 cells were grown in 24-well cell-culture trays. Each sample dilution was used to inoculate four wells using 0.1 mL of sample per well. The trays were incubated at 37°C for one hour with gentle rocking every 15 min. They were then placed in a 5% CO_2 incubator at 37°C and were examined daily for CPE for up to 14 days. Typically, CPE manifested itself as cell enlargement, rounding and the formation of grape-like clusters (Payment & Trudel 1993). The Karber $TCID_{50}$ method was used to quantify the viral concentration of each sample, as follows:

$$TCID_{50} = 10^{-(\Delta - \alpha(S-0.5))}$$

where: Δ = the $\log_{10}$ of the most diluted sample with 100% infectivity, α = the log dilution factor and S = the summation of the normalized positive infections, including both the last 100% and the first 0% infectious dilutions.

For the plaque assay, BGMK cells were cultured to confluency in 25-cm^2 flasks. The samples were added at a volume of 1 mL to the cells. The flasks were incubated at 37°C for 1 hour with gentle rocking every 15 min. After incubation, 4 mL of 1:1 plaque assay medium (1 × MEM supplemented with 2% FBS) 1.0% agarose overlay was added to each of the flasks. The flasks were then incubated at 37°C in a 5% CO_2 incubator for 48 hours. The agar overlay was removed and the cells were fixed using ethanol. The cells were then stained to allow visualization of plaques using a solution of 8.0% (wt./vol.) crystal violet and 20.0% (vol./vol.) ethanol in nanopure water.

IFA was used as the standard detection method for *Cryptosporidium* oocysts (USEPA 2005). All assays were performed in duplicate, and positive and negative controls were included in each set of assays. For IFA, 100-μL aliquots were serially diluted (10-fold) in 1 × PBS. Oocysts were stained with fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies against *Cryptosporidium* (Hydrofluor; Strategic Diagnostics Inc., Newark, DE), and enumerated by epifluorescence microscopy at 200 × magnification (BX-60; Olympus Optical Co., Ltd, Tokyo, Japan).

Real-time qPCR using TaqMan probe

In preparation for qPCR, the viral DNA/RNA from a 3-mL sample was purified with the QIAamp DNA/RNA mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The oocyst DNA was extracted using eight freeze–thaw cycles and was subsequently purified with the QIAamp DNA mini kit, as described previously (Ryu *et al.* 2008). The eluates were stored at − 20°C until used in the qPCR assay. The qPCR assay was performed with an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). PCR primers and a TaqMan probe specific for the VP1 gene of CoxB6 and Echo12 and the hexon gene of Ad4 were designed using Primer Express Software (Applied Biosystems) (Table 1). PCR primers and a TaqMan probe specific for the *C. parvum* hsp70 gene were used, as described by Di Giovanni & LeChevallier (2005).

The reaction for Ad4 (DNA virus) and *C. parvum* was performed in 20-μL volume containing 10 μL of 2 × TaqMan universal PCR master mix (Eurogentec, San Diego, CA), 300 nM of each primer, 200 nM TaqMan

Table 1 | Summary of oligonucleotide primers and probes for TaqMan qPCR

Enteric viruses	Primer or probe/position (5′–3′)*	Sequence†	T_m (°C)
Echovirus type 12	Forward primer (2523–2542)	5′-GAGCATCCCCGCACTAACAG-3′	59
	Reverse primer (2606–2587)	5′-TTCACATGACGGGTTTGCAT-3′	58
	Probe (2580–2555)	5′ FAM-CCCAGGAACCACCTGAGATGTATGCC-TAMRA 3′	68
Coxsackievirus B6	Forward primer (2455–2472)	5′-TGGAGGGCGCCATAGAGA-3′	59
	Reverse primer (2566–2549)	5′-GAGGTGTGGCCCGTTTCC-3′	60
	Probe (2500–2476)	5′ FAM-ATAGTGTCAGCGACCCGTGCAATGG-TAMRA 3′	69
Adenovirus type 4	Forward primer (19794–19816)	5′-CATGGACAACGTAAATCCCTTCA-3′	59
	Reverse primer (19866–19849)	5′-GTTGCCCAGGAGCATGGA-3′	60
	Probe (19818–19835)	5′ FAM-CCACCACCGCAATGCGGG-TAMRA 3′	68

*Position based on GenBank accession numbers: X77708 (VP1 gene of Echovirus type 12), AF105342 (VP1 gene of Coxsackievirus B6) and AY458656 (hexon gene of Adenovirus type 4).
†FAM, 6-carboxyfluorescein, fluorescence reporter dye; TAMRA, 6-carboxytetramethylrhodamine, fluorescence quencher dye.

probe and 2 μL of DNA template. Amplification conditions were as follows: initial denaturation at 95°C for 10 min followed by 40 (for Ad4) or 50 (for *C. parvum*) cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min. For CoxB6 and Echo12 (RNA viruses), the reaction was performed in a 20-μL volume containing 10 μL of 2 × one-step reverse transcriptase (RT) TaqMan PCR master mix with 0.1 μL of 50-U/μL EuroScript RT (Eurogentec, San Diego, CA), 300 nM of each primer, 200 nM TaqMan probe and 2 μL of RNA template. Amplification conditions were as follows: reverse transcription at 48°C for 30 min, RT inactivation and initial denaturation at 95°C for 10 min and 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min.

The fluorescent intensity was measured at the annealing phase of each cycle, and a threshold cycle (C_T) value for each sample was determined by the point at which the fluorescence exceeded a threshold limit. A qPCR standard curve was generated by plotting C_T values against the number of each virus ($TCID_{50}$ or PFU/ml) corresponding to serially diluted virus DNA/RNA standards. For *C. parvum*, DNA standards were prepared from oocyst DNA, extracted as described above. Amplification efficiencies of PCR were calculated using the following equation:

$$AE = -1 + 10^{(-1/\text{slope})}$$

where: a slope is given from a standard curve for each target microorganism. In addition to positive and negative controls, purification control experiments were performed to evaluate the level of PCR inhibition (USEPA 2004). Briefly, 3-mL water samples were collected from unseeded jar test experiments. The samples were purified using the QIAamp DNA/RNA mini kit and were then spiked with the DNA/RNA standards to test for PCR inhibition.

Chemical parameter analysis

The samples were analyzed for pH, turbidity, DOC and ultraviolet absorbance at a wavelength of 254 nm (UV_{254}), all of which may affect the efficiency of coagulation. The pH was measured using a Mettler (Columbus, OH) pH meter. Turbidity was measured using a Hach (Loveland, CO) model 2100P turbidimeter. For DOC determination, samples were filtered using 1.2-μm Whatman (Middlesex, UK) GF/C glass microfiber filters, acidified using 1 N HCl and analyzed using a Shimadzu 5050A (Kyoto, Japan) Total Organic Carbon Analyzer. A Shimadzu Multispec 1501 was used to measure UV_{254}

Data presentation and statistical analysis

Log removal or percent removal of microorganisms (L) is defined by the following equation:

$$L = -\log_{10}\left(\frac{N_d}{N_0}\right) \quad \text{or} \quad \left(1 - \frac{N_d}{N_0}\right) \times 100$$

where: N_0 and N_d are the initial concentration and the concentration of microorganisms after treatment, respectively.

L values were calculated as the average of at least four replicates of qPCR and duplicates of IFA.

Microbial removals determined using the two different detection methods were fitted by regression analysis according to the following equation:

$$P = -k \times S$$

where: k is the approximate coefficient, and P and S are the removal values calculated by qPCR and standard assays, respectively.

SPSS (Chicago, IL) Version 13.0 statistical software was used for the analyses at a significance level of $\alpha = 0.05$. An analysis of variance (ANOVA) test was used to test the equality of means. The Levene statistic was computed to test the ANOVA assumption of equal variances. The Welch test of equality of means was used for data not satisfying the equal variance assumption. A Pearson correlation coefficient (R) with a two-tailed P value was calculated for cross-correlations between the removal of oocysts, turbidity, DOC and UV_{254}. P values of < 0.05 were considered significant.

RESULTS AND DISCUSSION

Validation of qPCR for microbial removal studies

A qPCR standard curve was generated by plotting threshold cycle (C_T) values against the number of target microorganisms corresponding to five serially diluted DNA/RNA standards ($R^2 = 0.99$ for enteric viruses, $R^2 = 0.98$ for *C. parvum*). Each point represents the mean C_T value from four separate PCR amplifications (Table 2). In order to evaluate assay sensitivity, amplification efficiencies of PCR were calculated as described previously. The efficiencies for all target microorganisms with the exception of *C. parvum* met the efficiency criteria in the range of 90 to 110% (Table 2). The primers and probe used for *C. parvum* in this study were originally designed for environmental monitoring studies, considering high specificity (Di Giovanni & LeChevallier 2005). However, sensitivity may be more critical for removal studies since specific strains of microorganisms are generally used. Further study is needed to develop a new set of primers and probe for *C. parvum* detection in removal studies. The results of positive and negative controls were acceptable for every PCR reaction, and no significant level of PCR inhibition from testing water was observed ($P > 0.1$).

Table 2 | The threshold cycle (C_T) values against the number of target microorganisms (N) for qPCR standard curves

N*	Coxsackievirus	Echovirus	Adenovirus	*C. parvum*
10^5	19.7	15.8	19.6	29.1
10	35.1	28.7	32.7	42.3
Amplification efficiencies	99.3%	93.8%	98.8%	118.9%

*Units of the number of target microorganisms: PFU for coxsackievirus and echovirus, $TCID_{50}$ for adenovirus and oocysts for *C. parvum*.

Note: The C_T values for the lowest and highest dilutions of 5 DNA/RNA standards are provided in the table. Approximately 3-unit increment of C_T value for each standard was obtained (data not shown).

In terms of performing virus removal studies, particularly using advanced treatment processes such as enhanced coagulation or membrane filtration, a relatively high titer of challenging viruses should be spiked. In general, the titer of the viruses is estimated using $TCID_{50}$ obtained from a cell-culture analysis. It is hypothesized that qPCR measurements are greater than $TCID_{50}$ because qPCR measures non-infectious viruses as well as infectious ones. In this study, qPCR detected up to 10^{-4} $TCID_{50}$ of Ad4 (data not shown), resulting in over four orders of magnitude higher titer estimated by qPCR than by a cell-culture assay. Therefore, qPCR could provide for more rigorous investigation of physical removal of viruses in the high removal range. In addition, this cell-free method could be applied to extreme water conditions, e.g. low pH and high chemical concentrations, where enteric viruses are treated by dual processes such as physical removal and chemical disinfection. To elucidate virus removal mechanisms (i.e. physical removal and/or inactivation) in combined treatment processes, further investigation using both cultural-based and molecular techniques needs to be performed.

Cell-culture assays require separate experiments for some enteric viruses since they infect the same cells. In this study, we demonstrated the applicability of qPCR for the simultaneous quantification of coxsackievirus and echovirus. In addition, poliovirus was added for evaluating cross-reactivity among the target viruses. The results demonstrated that the TaqMan probes designed for this study prevented cross-reactivity by amplifying only the target virus (data not shown). In other words, qPCR

enables the simultaneous quantification of multiple enteric viruses without cross-reactivity, thereby enhancing its applicability for virus removal studies. Also, this molecular technique can be used for removal studies of non-cultivatable enteric viruses. For example, until recent advances using low-shear rotating wall vessel bioreactors, *in-vitro* cell-culture systems for human norovirus (also included on the CCL) were unavailable (Straub *et al.* 2007). To date, only a few removal studies using norovirus surrogates such as bacteriophages and feline calicivirus have been reported (Abbaszadegan *et al.* 2007, 2008). Cell-free molecular techniques such as qPCR may feasibly be applied to studies of human norovirus removal.

Comparison of qPCR and standard detection methods

Based on the qPCR standard curve, relative quantification was used to estimate microbial removals during water treatment processes. The removal efficacies of three CCL viruses (Ad4, CoxB6 and Echo12) by enhanced coagulation were determined using two detection methods: qPCR and cell-culture assays (Figure 1). There were significant correlations between the two methods for log removal of coxsackievirus ($R^2 = 0.704$, $n = 9$) and echovirus ($R^2 = 0.809$, $n = 9$). Also, the removal efficacies determined by the two methods showed an approximate 1:1 (qPCR:cell culture) correlation for log removal of coxsackievirus (1.04:1) and echovirus (1.17:1). These results suggest that

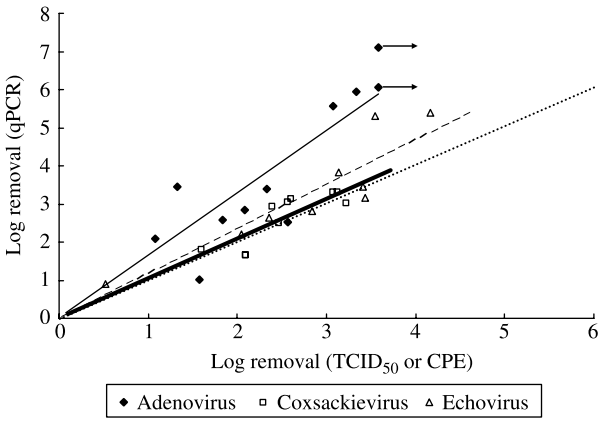

Figure 1 | Comparison of virus removal efficiencies determined by qPCR and cell-based infectivity assays. Each point on the figure represents the mean value from at least four replicate samples. → indicates that a sample is below the detection limit of the cell-based infectivity assay, which excluded those data from the curve fitting.

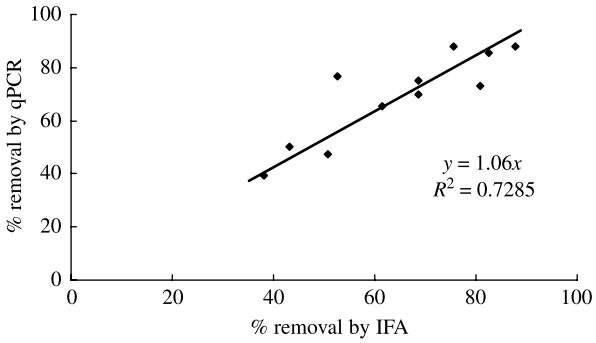

Figure 2 | Comparison of oocyst removal efficiencies determined by qPCR and IFA. Each point on the figure represents the mean value from at least four replicate samples for qPCR and from duplicates for IFA.

qPCR is a satisfactory alternative to cell-culture assays for enteric virus removal studies. The removal efficacy of adenovirus showed a significant correlation ($R^2 = 0.614$, $n = 11$), but the estimation of log removal determined by qPCR was approximately 50% higher than cell culture (i.e. 1.54:1 correlation). One possible explanation is the relatively conservative determination of CPE for adenovirus compared to a PFU assay for the other viruses, thereby resulting in an underestimation of log removal. Further study is needed to elucidate the difference between the two methods for adenovirus.

For *C. parvum* oocysts, the removal efficiencies determined using the two detection methods (qPCR and IFA) were fitted using regression analysis (Figure 2). A significant correlation between percent removals calculated using qPCR and IFA was observed ($R^2 = 0.729$, $n = 11$). Also, the removal efficacies determined by the two methods showed an approximate 1:1 correlation for percent removal of *C. parvum* oocysts (i.e. qPCR:IFA = 1.06:1). These results indicate that qPCR is a satisfactory alternative to IFA for oocyst removal studies.

Oocyst removal by enhanced coagulation

The physical removal of coxsackievirus and echovirus (Mayer *et al.* 2008) and adenovirus (Abbaszadegan *et al.* 2007) using enhanced coagulation was presented in the companion studies. Here, the removal efficacy of *C. parvum* oocysts is described. In coagulant dose optimization experiments, 25% or greater DOC removal was achieved using coagulant doses of 40 mg/L or greater $FeCl_3$, which satisfies

the USEPA guidelines for enhanced coagulation (USEPA 1999). Increasing the $FeCl_3$ concentration from 20 to 120 mg/L (resulting in a pH range of 6.5 and 8.0) resulted in a steady increase in the percent removal of *C. parvum* oocysts, ranging from 65 to 88%. These removals compare favorably with those reported by Xagoraraki & Harrington (2004), whereas other studies showed higher oocyst removals (Keegan *et al.* 2008). Edzwald & Kelly (1998) reported 1.2 log removal (94%) with 20 mg/L alum at pH 6.5. Oocyst removals reported by Harrington *et al.* (2001) ranged from 0.1 log (21%) to 2.9 log (99.87%) with alum doses ranging from 20 to 40 mg/L and pH ranging from 6.0 to 7.6.

A variety of factors may affect oocyst removals, including differences in source water quality, different types of coagulants, spiking levels of oocysts, different isolates of oocysts and surface characteristics of oocysts. For example, ferric chloride was used in this study, while alum coagulation was used for the above-cited studies. Bustamante *et al.* (2001) suggested that the mechanisms for oocyst removal by ferric chloride and alum coagulation may differ (i.e. a combination of charge neutralization and sweep flocculation for alum coagulation and mainly sweep flocculation for ferric chloride coagulation). Also, alum flocs are typically larger than ferric chloride flocs, suggesting that coagulation with alum may achieve greater removal of oocysts. In addition, Xagoraraki & Harrington (2004) used viable oocysts (as was done in this study), whereas formaline-inactivated oocysts were used in other studies. Formaline treatment alters the surface characteristics of oocysts (Brush *et al.* 1998; Kuznar & Elimelech 2005), and may consequently affect oocyst removal. Keegan *et al.* (2008) reported that oocysts purified and preserved with surfactants and antibiotics were characterized by a relatively low zeta potential, which contributes to lower removal efficacy.

The pH optimization jar tests were performed using a dose of 40 mg/L $FeCl_3$ and pH adjustment (down to 5.5 with decrements of 0.5). Less than 25% removal of DOC was achieved for all pH adjusted samples (data not shown), which failed to meet the USEPA enhanced coagulation guidelines (USEPA 1999). Oocyst removals demonstrated a consistent decrease in response to reductions in pH and slight improvement at pH of 5.5 (data not shown). This result may support the hypothesis that the main mechanism for oocyst removal by ferric chloride is sweep flocculation (a function of coagulant dose), rather than charge neutralization (a function of pH) (Bustamante *et al.* 2001). Further study is needed to elucidate the mechanisms of oocyst removals as a function of pH.

Correlation analyses were performed to determine if chemical parameters such as turbidity, DOC and UV_{254} were good indicators for the removal of *C. parvum* oocysts by coagulation. The results showed a strong correlation between the removal of oocysts and turbidity ($R = 0.81$), whereas the other combinations (i.e. oocysts vs. DOC, oocysts vs. UV_{254}) resulted in poor correlations ($R < 0.15$).

CONCLUSION

The results of control experiments including PCR inhibition controls and cross-reactivity controls demonstrate that qPCR is a promising relative quantification technique, particularly for microbial removal studies. The significant correlation between microbial removals determined by qPCR and standard detection methods and an approximate 1:1 correlation suggest that qPCR could be a satisfactory alternate for quantification in microbial removal studies. In coagulant dose optimization experiments, the USEPA criterion was satisfied by removing more than 25% of DOC at coagulant doses of 40 mg/L $FeCl_3$or greater. Under these enhanced coagulation conditions, removal of *C. parvum* oocysts ranged from 65 to 88%, which compares favorably with other research. The removal of turbidity showed a strong correlation with oocyst removal, suggesting that water turbidity measurement can be a useful tool to estimate the removal of oocysts by enhanced coagulation.

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