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Quantification of viable *Giardia* cysts and *Cryptosporidium* oocysts in wastewater using propidium monoazide quantitative real-time PCR

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Abstract

The quantification of viable *Giardia* cysts and *Cryptosporidium* oocysts in wastewater with a quantitative real-time PCR incorporating pretreatment of the samples with propidium monoazide (PMA) was evaluated. The reduction in *Giardia duodenalis* cysts detected was 83.2%, 89.9%, 98.2%, or 97% with PMA–qPCR assays amplifying β-giardin (75-bp), triosephosphate isomerase (77-bp), glutamate dehydrogenase (133-bp), or β-giardin (143-bp) genes, respectively. When qPCR assays that produced larger amplicons were used, the exclusion of dead cysts was more effective. The PMA treatment of *Cryptosporidium* oocysts plus/minus heat treatment abolished the fluorescent signal for dead oocysts with PMA-qPCR assay amplifying *C. parvum* (150-bp) oocyst wall protein (*COWP*) gene. The PMA-qPCR 143-bp β-giardin assay for *Giardia* and the PMA-qPCR 150-bp COWP assay for *Cryptosporidium* failed to detect dead oo(cysts) when PBS and tertiary effluent wastewater were spiked with concentrations of 10^3 or 10^2 dead oo(cysts), respectively. Therefore, these assays are suitable for the detection of viable parasites that are typically present in tertiary wastewater effluents at concentrations of $< 10^3$ oo(cysts)/l.

Keywords *Cryptosporidium*, *Giardia*, PMA, qPCR, wastewater, viable, oocyst, cyst

Introduction

Giardia duodenalis and *Cryptosporidium* are common food- and water-borne protozoa that affect humans and a wide range of domestic and wild animals (Fayer 2004). These parasites are among the major causal agents of diarrheal disease in humans and animals world-wide, and can even potentially shorten the lifespans of immunocompromised hosts (Reynolds et al. 2008). *Cryptosporidium* oocysts and *Giardia* cysts derived from contaminated feces flowing into wastewater treatment systems are often found in the raw wastewater of wastewater treatment plants in some regions of the world (Cacciò et al. 2003; Alonso et al. 2011; Liu et al. 2011; Guy et al. 2003; Sulaiman et al. 2004).

The introduction of molecular techniques, particularly those based on the amplification of nucleic acids, has provided researchers with highly sensitive and specific assays for the detection and quantification of protozoans. One very effective molecular tool is quantitative PCR (qPCR), but a serious limitation of the technique is that it cannot discriminate between viable and nonviable protozoa (Fittipaldi et al. 2011). To overcome this problem, qPCR has been combined with propidium monoazide (PMA) or ethidium monoazide treatment to effectively distinguish between viable and nonviable bacteria (Nocker et al. 2006; Guy et al. 2006; Agusti et al. 2010), protozoa (Fittipaldi et al. 2011), viruses (Fittipaldi et al. 2010), and fungi (Vesper et al. 2008). PMA is a chemically modified version of propidium iodide, with an azide group added to the phenanthridine ring, allowing its chemical cross-linkage to organic molecules during short exposure to bright visible light (Nocker et al. 2006). The photolysis of PMA converts the azide group into a highly reactive nitrene radical, which can react with any organic molecule in its proximity, including DNA. In this bound state, the DNA cannot be amplified by PCR (Nocker and Camper 2009). The photoactivation of PMA is a key factor in the successful application of PMA–qPCR, but although this technique has been used in multiple studies, there has been little standardization of the photoactivation step.

Until now, many studies have used high-power halogen lamps (500–750 W) for this photoactivation. However, with these lamps, the samples must be placed on ice to prevent heating and the loss of sample, and positioned at a distance of 20–30 cm to separate the sample from the light source (Nocker and Camper 2009). A dedicated device to photoactivate PMA was recently developed using light-emitting diode (LED) technology (Fittipaldi et al. 2012). With this device, sample overheating is avoided, so possible damage to the cell membrane during photoactivation is reduced or eliminated, and the simplicity and efficiency of the method is enhanced (Fittipaldi et al. 2012). Therefore, the use of LED lamps could be an important step toward the standardization of the photoactivation step.

Only a handful of studies have used PMA–qPCR to quantify viable *Cryptosporidium* oocysts and no study has yet quantified viable *Giardia* cysts. Brescia et al. (2009) demonstrated the use of PMA before conventional PCR treatment (CryptoPMA–PCR) to specifically detect and genotype viable *Cryptosporidium* oocysts. Liang and Keeley (2012) evaluated the quantification of viable *C. parvum* oocysts in water when the *hsp*70 gene was targeted with PMA–qPCR. High-power halogen lamps were used to photoactivate PMA in these studies. Recently, Agulló-Barcelló et al. (2013) demonstrated the use of the LED device to photoactivate PMA-treated *Cryptosporidium* oocysts for quantification, targeting the 18S rRNA gene.

The aim of our study was to determine whether qPCR combined with PMA photoactivation (PMA–qPCR) using the LED technology is a good alternative technique with which to quantify viable *Giardia* cysts and *Cryptosporidium* oocysts in effluent wastewater samples.

Material and methods

Inactivation treatment

Viable *C. parvum* oocysts (Iowa isolate) and *G. duodenalis* (H3 isolate) cysts were obtained from Waterborne (New Orleans, LA, USA). Following centrifugation at 13,000 × g for 1 min, the (oo)cysts were resuspended in 100 µl of 0.1 M phosphate-buffered saline (PBS, pH 7.2). The viable (oo)cyst suspensions were diluted to concentrations of approximately $5x10^4$ live (oo)cysts, and then heat-treated in a laboratory heat block at 70 °C for 30 min. Aliquots with the same concentration of (oo)cysts, but without the inactivation treatment, were used as the controls.

PMA treatment of viable and nonviable *Cryptosporidium* oocysts and *Giardia* cysts

Briefly, PMA (Biotium, Inc., Hayward, CA, USA) was dissolved in 20% dimethyl sulfoxide (Sigma Aldrich) to create a stock concentration of 2 mM, aliquoted in 20 µl volumes, and stored at −20 °C in the dark for no longer than 2 months. A volume of 5 µl of the 2 mM PMA stock solution was rapidly added to microcentrifuge tubes containing approximately 10^4, 10^3, or 10^2 live or heat-treated oo(cysts) suspended in 61.6 µl of PBS, resulting in a final concentration of 150 µM PMA. The resultant (oo)cyst suspensions were incubated for 5 min at room temperature in the dark to allow the PMA to enter into the (oo)cysts with compromised or damaged walls. The samples were then photoactivated for 15 min using a LED light source (λ = 464–476 nm, 60 W; PhAST Blue PhotoActivation System for Tubes, GenIUL, Barcelona, Spain) before DNA extraction. After the oocysts were cross-linked by photoinduction, they were pelleted by centrifugation at 13,000 × g for 1 min. The supernatant was discarded and the pellet was resuspended in 180 µl of ATL buffer (Qiagen, Hilden,

Germany), and stored at −20 °C until the DNA was extracted for use in qPCR. Aliquots of PBS spiked with the same concentrations of live or heat-killed oo(cysts), without PMA treatment, were used as the controls.

Genomic DNA extraction

DNA was extracted from (oo)cysts with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The (oo)cysts suspended in 180 µl of ATL lysis buffer (Qiagen) were subjected to 15 freeze–thaw cycles (1 min in liquid nitrogen and 1 min at 65 °C per cycle), and 20 µl of proteinase K was added to each tube. The tubes were incubated overnight at 56 °C. The DNA was purified through the column according to the manufacturer's protocol and eluted from the column with 100 µl of TE buffer.

Quantitative PCR

The qPCR and data analysis for *Cryptosporidium* were performed with the LightCycler 2.0 PCR system (Roche, Barcelona, Spain), with the qPCR cycling conditions described in Table 1. The *Giardia* primer and probe sequences were used as described in the original publications (Table 1), except that the forward primer for glutamate dehydrogenase (*gdh*) gene detection was modified. An additional A was added in the 5′ end of the sequence after the alignment of multiple *gdh* sequences from GenBank. The qPCR for *Giardia* was performed on an HRM 6500 Rotorgene instrument (Corbett, Australia). The Agilent Brilliant III Fast QPCR Kit (Agilent Technologies, Ontario, Canada) was used for the probe-based qPCR assays (75-bp β-giardin, 143-bp β- giardin [OCU36/39]), and the Agilent Brilliant II

SYBR QPCR Master Mix was used for the melt assays [605-bp triosephosphate isomerase (*tpi*); 133-bp glutamate dehydrogenase (*gdh*)]. Each 25 µl reaction included 5 µl of DNA template and 300 ng/µl BSA (fraction V; Sigma, St. Louis, MO, USA). A no-template control was included in every assay, and no cycle threshold (Ct) values were consistently obtained after 50 cycles of PCR. The qPCR assays were performed with duplicate samples. The results presented are the means of these assays and are expressed as (oo)cysts/ml.

Detection of viable and nonviable (oo)cysts in environmental samples

Flat-sided 12 ml Leighton tubes containing 10 ml of oo(cyst)-free tertiary-treated wastewater effluent (UV disinfection) were spiked with 10^4, 10^3, or 10^2 live or heat-killed oo(cysts). The (oo)cysts were separated from the sample matrix using Dynabeads immunomagnetic separation (DynaBeads® GC Combo kit; Invitrogen-Dynal A.S., Oslo, Norway), according to the manufacturer's guidelines. After separation from the sample matrix, the oocyst–bead complexes were resuspended in 61.6 µl of 0.1 M PBS (pH 7.2) and treated with PMA as described above. Oo(cyst)-free wastewater spiked with aliquots of the same concentrations of live or heat-killed oo(cysts), without PMA treatment, were used as the controls. After photoinduced cross-linking, the oocysts were pelleted by centrifugation at 13,000 × g for 1 min. The supernatant was discarded and the pellet was resuspended in 180 µl of ATL buffer and stored at −20 °C until DNA extraction using the QIAamp DNA Mini Kit, for use in qPCR. The qPCR assays were performed with duplicate samples. The results presented are the means of these assays and are expressed as (oo)cysts/ml.

Viability staining

In our study, (oo)cysts were labelled in suspension, and subsamples of the suspensions mounted onto microscope slides and viewed without drying, as recommended by Robertson et al. (2014). Live or heat-killed (oo)cysts suspended in 100 µl of PBS were incubated with 25 µM PMA for 10 min and stained with the Aqua-Glo™ G/C Direct fluorescent antibody staining kit, according to the manufacturer's instructions (Waterborne) and combined with 2 µM SYTO 9 nucleic acid stain. The (oo)cysts stained with fluorescein isothiocyanate (FITC)-conjugated antibody and SYTO 9 were observed with an FITC filter set, whereas (oo)cysts with PMA-labelled DNA were observed with a rhodamine filter set. It has previously been shown that the nucleic acid dye SYTO 9 stains heat-inactivated oocysts but not viable oocysts (Belosevic et al. 1997). Microscopic examinations were performed with an Olympus BX 50 epifluorescence microscope (Olympus BX50, Tokyo, Japan) equipped with bright-field, differential interference contrast, and epifluorescence optics.

Results

Viability staining

After PMA treatment, the live (oo)cysts remained impermeable to PMA and did not stain red (Figs 1C and 2C), whereas the heat-killed (oo)cysts stained bright red with PMA (Figs 1F and 2F). The dead oocysts stained yellow–green with SYTO 9 (Fig. 1E).

Quantitative PMA–PCR

The results for the PMA treatment of *Giardia* cysts plus/minus heat differed from those observed for *Cryptosporidium*. qPCR amplification using the β-giardin qPCR assay, which amplifies a 75-bp product, showed an 82.3% reduction in the fluorescent signal, and therefore

detected a small proportion of dead cysts. Because amplicon size is important in PMA–qPCR, we compared published qPCR assays specific for assemblage B *G. duodenalis* that generated DNA amplicons of various sizes (Table 1). The reduction in cyst detection was 83.2%, 89.9%, 98.2%, and 97% for the 75-bp β-giardin, 77-bp *tpi*, 133-bp *gdh*, and 143-bp β-giardin qPCR assays, respectively (Fig. 3). No complete elimination of the target signal was achieved when we analyzed samples at a concentration of 10^4 cysts suspended in PBS with these qPCR assays. The application of PMA–qPCR to either PBS (Fig. 4A) or wastewater samples (Fig. 4C) spiked with 10^4 cysts resulted in the complete elimination of the qPCR signal for 605-bp *tpi* amplicon but the incomplete elimination of the qPCR signal for the 143-bp β-giardin amplicon. In contrast, when the samples were spiked with lower concentrations of cysts (10^3 or 10^2), both the 605-bp *tpi* and 143-bp β-giardin PMA–qPCR signals were completely eliminated (Fig. 4).

Although the 605-bp *tpi* qPCR assay provided reliable quantification, it must be noted that the efficiency of this assay was low, ranging from 0.6617 to 0.787. In an attempt to improve the efficiency of this assay, we tested three different SYBR-based detection qPCR master mixes [Brilliant II SYBR® Green QPCR Master Mix (Agilent); Quantitech SYBR® Green PCR Kit (Qiagen); and FastStart SYBR Green Master (Roche)], and an EvaGreen®-based master mix [Type-it HRM PCR master mix (Qiagen)]. The SYBR-based master mixes resulted in efficiencies of 0.781, 0.769, and 0.693 for the Agilent, Qiagen, and Roche assays, respectively. The efficiency of the EvaGreen-based assay was 0.697. Although the use of different master mixes did not improve the efficiency of the assay, the sensitivity of detection was good for all the master mixes when the DNA was extracted from cysts suspended in PBS. It should be noted that the efficiencies of the qPCR probe-based assays and SYBR-based assays that produced amplicons of ≤ 143 bp were consistently higher than those producing longer amplicons, ranging from 0.865 to 0.984.

The results of the PMA treatment of *Cryptosporidium* oocysts plus/minus heat treatment resulted in the complete abolition of the fluorescent signal for dead oocysts with PMA-qPCR assay amplifying C. parvum (150-bp) oocyst wall protein (*COWP*) gene (Fig. 5). In our study, live oocysts, but not dead oocysts, were detected in tertiary effluent wastewater samples spiked with live or dead *Cryptosporidium* oocysts(Fig. 5).

Discussion

Staining with vital dyes has long been used to assess the viability of *Cryptosporidium* oocysts (Belosevic et al. 1997) and *Giardia* cysts (Smith and Smith 1989). Our results show that heat-killed oo(cysts) stained bright red with PMA. Parasites that can be stained with vital dyes, in particular propidium iodide (PI) or PMA, are considered nonviable, because this staining demonstrates a lack of cell-wall integrity (Robertson et al. 2014; Brescia et al. 2009).

qPCR of dead PMA-treated oocysts and live PMA-treated oocysts produced remarkably different results. The covalent binding of PMA to the DNA of heat-killed *Cryptosporidium* oocysts strongly inhibited qPCR amplification. Brescia et al. (2009) demonstrated that heat-killed oocysts are permeable to PMA, whereas live oocysts are impermeable to it. The PMA–qPCR assay was able to detect live oocysts in oocyst-free tertiary wastewater effluent samples spiked with live or heat-killed oocysts, whereas the DNA of heat-killed oocysts was not amplified. The presence of tertiary wastewater effluent concentrates did not seem to influence the performance of the *Cryptosporidium* qPCR–PMA assay. The concentrations of total solids in the tertiary wastewater effluents varied from 5 to 25 mg/l, with a mean value of 10 mg/l. Past reports have noted inconsistencies in the detection of oocyst viability with the qPCR–PMA method, attributed to the presence of total solids at concentrations of ≥ 680 mg/l, which might inhibit the cross-linking step in the PMA–DNA assay (Liang and Keeley 2012).

Amplicon size is another important factor in the PMA–qPCR assay (Contreras et al. 2011; Fittipaldi et al. 2012). Agulló-Barceló et al. (2013) demonstrated that PMA was more efficient at suppressing the fluorescent signal from dead oocysts when an 834-bp fragment of the 18S rRNA gene of *C. parvum* was targeted than when a 108-bp fragment was targeted. The 605-bp *TPI* gene target assay was adapted from the study of Sulaiman et al. (2003) and was originally designed to genotype *Giardia* with conventional PCR (Sulaiman et al. 2003). Longer sequences reduce the signal from dead cells more efficiently in the qPCR–PMA viability assay using LED photoactivation by maximizing the probability that at least one binding event will occur in the targeted region (Agulló-Barceló et al. 2013). However, it is known that PCR assays that generate larger amplicons can be less efficient than small-amplicon qPCR assays (Opel et al. 2010), as seen in our study. Furthermore, large-amplicon assays can be more sensitive to PCR inhibitors, resulting in lower efficiencies and lower detection in the presence of inhibitors (Opel 2010). Therefore, caution should be exercised in the use of long-amplicon qPCR assays for PMA–qPCR because they may result in the underestimation of risk because the numbers of live cells are underestimated.

The PMA concentration is also an important parameter in this assay and all the parameters are interlinked, so an optimized balance of parameters is required for the specific application of interest (Fittipaldi et al. 2012). Liang and Keeley (2012), using a final PMA concentration of 50 μM, demonstrated the suitability of the qPCR–PMA method targeting the *hsp*70 gene for evaluating the viability of *Cryptosporidium* oocysts after the disinfection of water with hydrogen peroxide. Agulló-Barceló et al. (2013) used a final PMA concentration of 50 μM to amplify two PCR products of 108 bp and 834 bp. The application of higher dye concentrations should also be considered. High PMA concentrations (such as 150 μM) have been used without significant cytotoxic effects on viable oocysts (Brescia et al. 2009). In our *Cryptosporidium* qPCR–PMA assay, the combination of PMA at a concentration of 150 μM,

LED photoactivation, and the amplification of the 150-bp *COWP* target gene in the qPCR assay strongly inhibited the signal from heat-killed oocysts, with no inhibition of the signal from viable oocysts.

In conclusion, LED-based photoactivation worked well in photoactivating PMA in both *Giardia* and *Cryptosporidium* oo(cysts). The 143-bp β-giardin qPCR assay for *Giardia* and the 150-bp *COWP* assay for *Cryptosporidium* detected no dead oo(cysts) when PBS and tertiary effluent wastewater were spiked with concentrations of 10^3 and 10^2. Therefore, these assays are suitable for the detection of parasites that are typically present at concentrations of $< 10^3$ oo(cysts)/l in tertiary wastewater effluents.

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Table
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Table 1. Primer–probe sets and cycling parameters for the *Giardia* and *Cryptosporidium* qPCR assays used in this study

Target	Primer and probe sequences (5'-3')[a]	Cycling parameters	Amplicon size (bp)	Reference
	Giardia duodenalis			
β-giardin gene	F:CATCCGCGAGGAGGTCAA R:GCAGCCATGGTGTCGATCT P:FAM/AAGTCCGCCGACAACATGTACCTAACGA/TAMRA	10 min at 95 ºC; 45 cycles of 10 s at 95 ºC, 8 s at 58 ºC and 3 s at 72 ºC	74	Guy et al. (2003)
TPI gene Assemblage B	F:GATGAACGCAAGGCCAATAA R: AAGAAGGAGATTGGAGAATC SYBR detection	10 min at 95 ºC; 45 cycles of 15 s at 95 ºC, 30 s at 59 ºC and 10 s at 72 ºC	77	Almeida et al 2010
TPI gene	F:AAATIATGCCTGCTCGTCG R:CAAACCTTITCCGCAAACC SYBR detection	5 min at 95 ºC; 45 cycles of 45 s at 95 ºC, 45 s at 50 ºC and 60 s at 72 ºC	605	Sulaiman et al. 2003
β-giardin gene Assemblage B	F:GGCCCTCAAGAGCCTGAAC R:GAGAAGGAGACGATCGCCC P:FAM/CGAGACAGGCATC/MGBNFQ	10 min at 95 ºC; 45 cycles of 15 s at 95 ºC, 60 s at 60 ºC	143	Baque et al 2010
GDH Assemblage B	F:CGATATTGGCGTCGGCGGT R:TGTGGCCTCTGGTCTGATAG SYBR detection	10 min at 95 ºC; 45 cycles of 15 s at 95 ºC, 30 s at 59ºC and 10 s at 72ºC	133	Almeida et al 2010[b]
	Cryptosporidium parvum			
C.*parvum* COWP gene	F:CAAATTGATACCGTTTGTCCTTCTG R:GGCATGTCGATTCTAATTCAGCT P:HEX/TGCCATACATTGTTGTCCTGACAAATTGAAT/BHQ	10 min at 95 ºC; 45 cycles of 10 s at 95 ºC, 8 s at 66 ºC and 6 s at 72 ºC	150	Guy et al. (2003)

[a]F, forward primer; R, reverse primer; P, probe; [b]the GDH R primer was modified from the original primer (see Material and Methods); FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; MGBNFQ, minor-groove-binding nonfluorescent quencher; HEX, hexachlorofluorescein; BHQ, black hole quencher.

Figure
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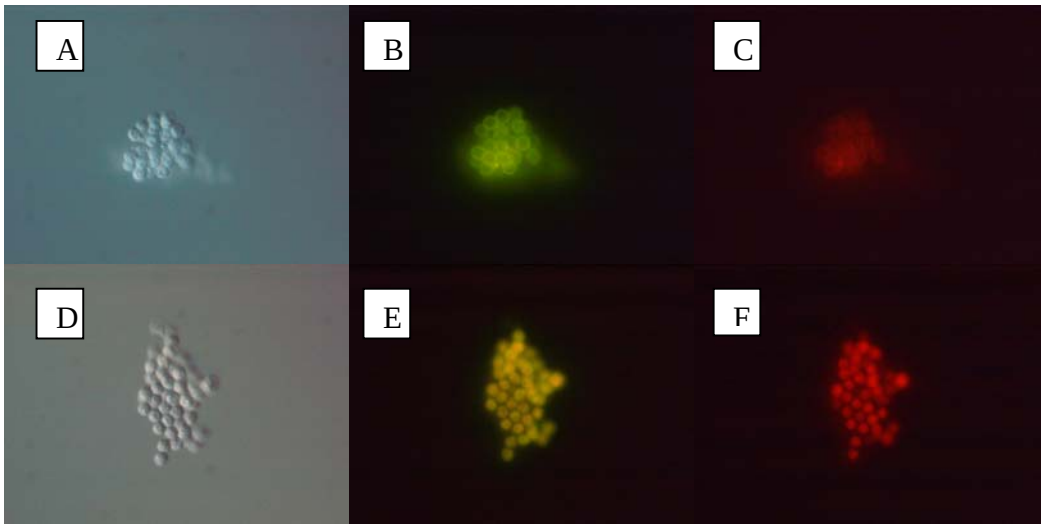


Fig. 1. Microscopic analysis distinguishing live from dead *C. parvum* oocysts. Live: A) DIC, B) SYTO 9 plus Aqua-Glo G/C, C) PMA. Heat-killed (70 ºC, 30 min): D) DIC, E) SYTO 9 plus Aqua-Glo G/C, F) PMA, 1,000X.

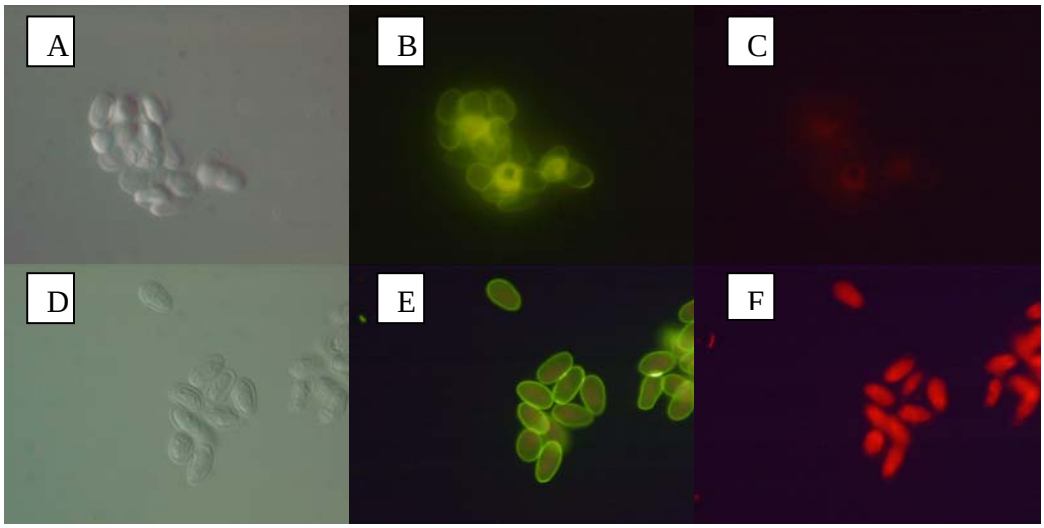


Fig. 2. Microscopic analysis distinguishing live from dead *G. duodenalis* cysts. Live: A) DIC, B) Aqua-Glo G/C, C) PMA. Heat-killed (70 ºC, 30 min): D) DIC, E) Aqua-Glo G/C, F) PMA, 1,000X.

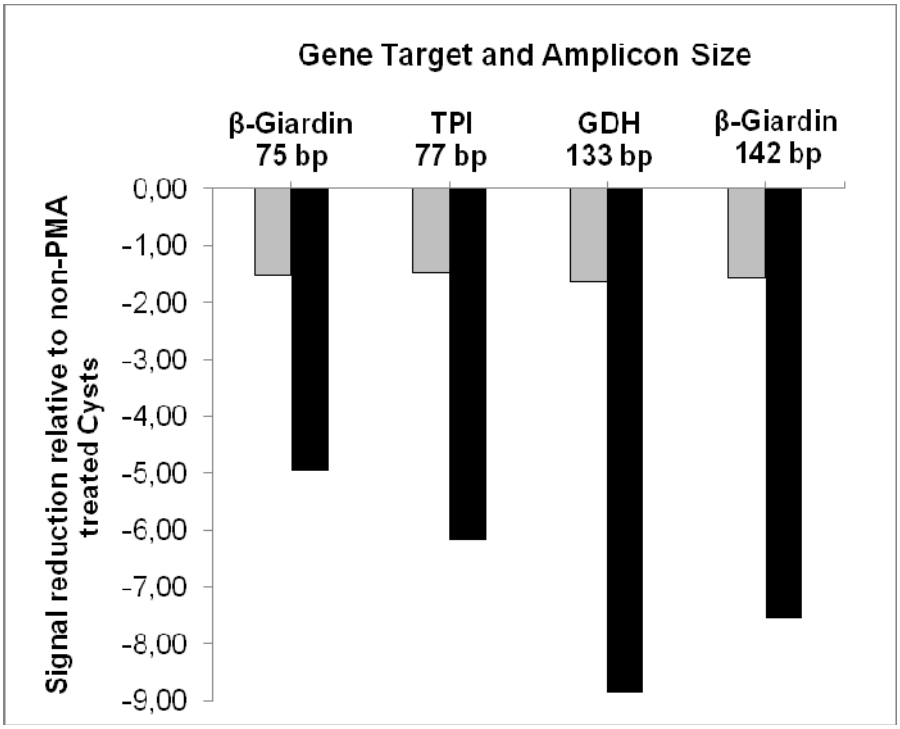


Fig. 3. Effect of amplicon size on PMA-qPCR signal reduction of live and dead *Giardia* cysts in four qPCR assays. Signal reduction values were derived by subtracting the Ct‘s of PMA-treated cysts from non PMA-treated cysts. Grey bars represent live cysts and black bars represent dead cysts.

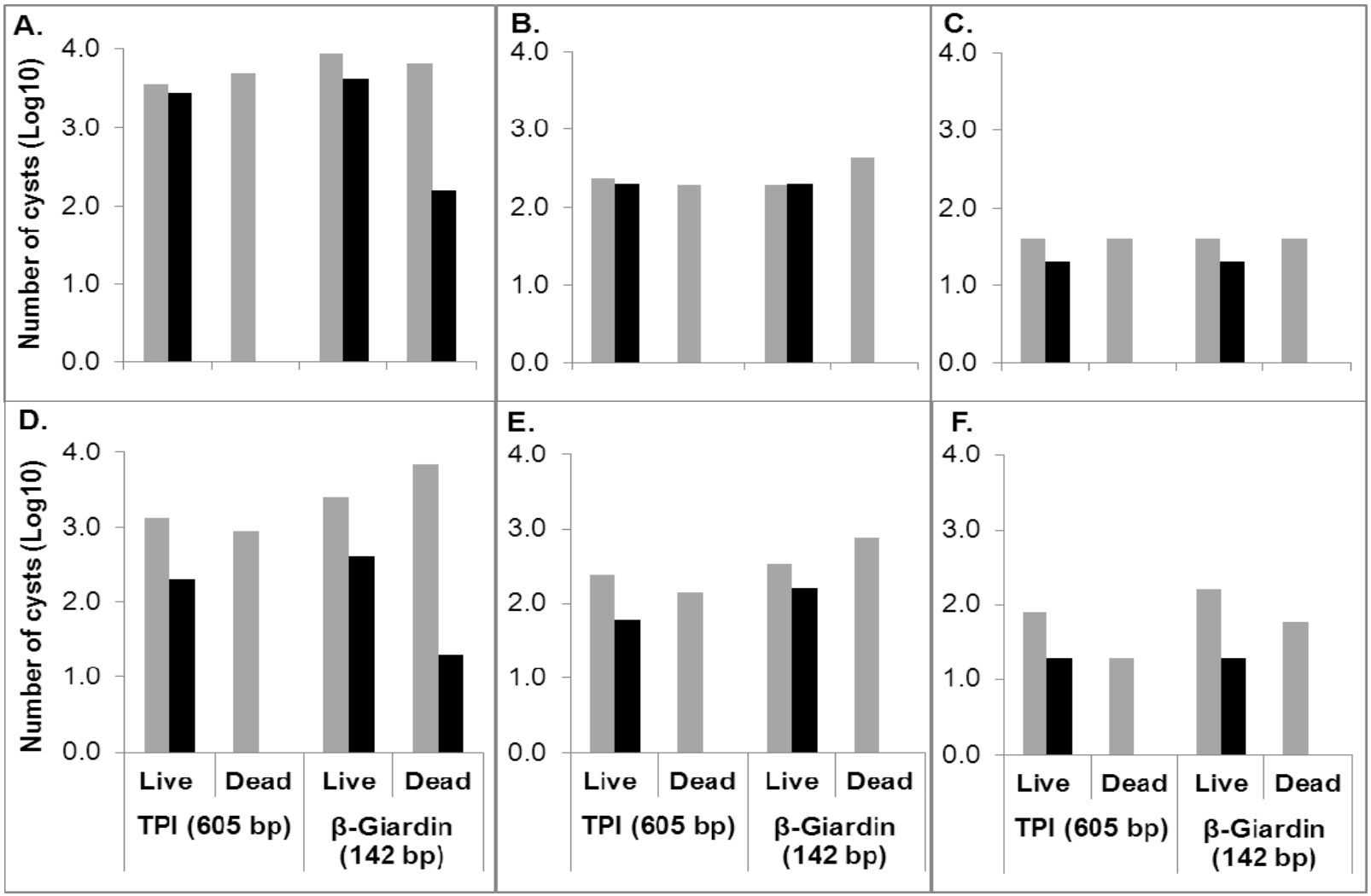
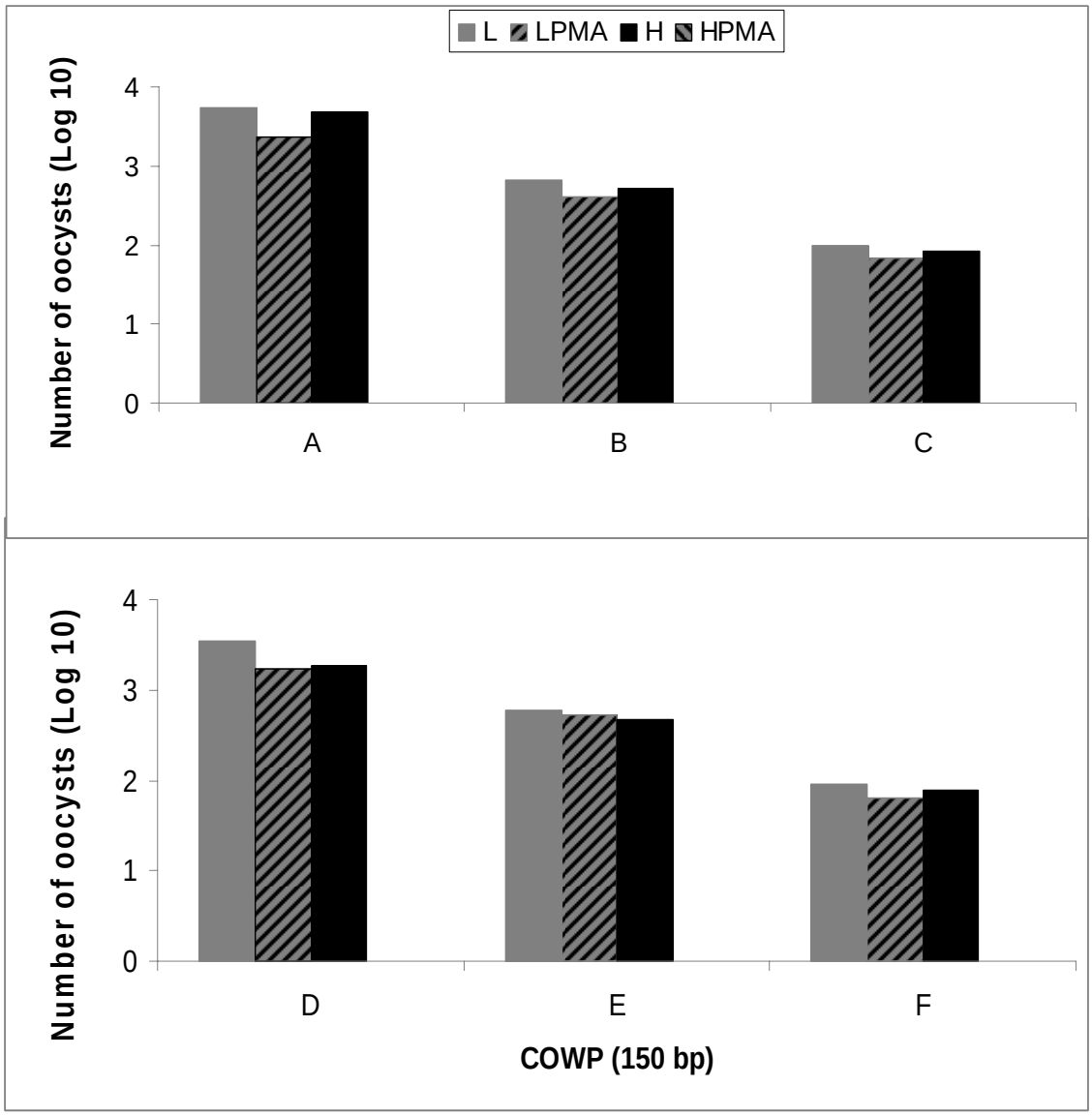


Fig. 4. Effects of different amplicon sizes on qPCR quantification of live and heat-killed (70ºC, 30 min) *Giardia* cysts in the presence or absence of PMA treatment. The bars represent the log number of cysts derived from qPCR in the absence (grey bars) or presence (black bars) of PMA. Cysts were spiked into either PBS (figures A, B, C) or raw wastewater (D, E, F), at three difference concentrations of cysts: 10^4 (A, D), 10^3 (B, E) and 10^2 (C, F).



L: Live oocysts non PMA treated; LPMA: Live oocysts PMA treated, H: Heat-killed oocysts non PMA treated; H: Heat-killed oocysts PMA treated

Fig. 5. QPCR quantification of live and heat-killed (70ºC, 30 min) *Cryptosporidium* oocysts in the presence or absence of PMA treatment. The bars represent the log number of cysts derived from qPCR in the absence (grey bars) or presence (black bars) of PMA. Oocysts were spiked into either PBS (figures A, B, C) or raw wastewater (D, E, F), at three difference concentrations of cysts: 10^4 (A, D), 10^3 (B, E) and 10^2 (C, F).