Title: CryptoPMA-PCR: A Molecular-Based Technique for Genotyping Viable *Cryptosporidium* Oocysts Using Propidium Monoazide to Prevent the Amplification of DNA from Non-Viable Oocysts

Running title: Detection of live *Cryptosporidium* oocysts

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ABSTRACT

Cryptosporidium is an important waterborne protozoan parasite that can cause severe diarrhea and death in the immunocompromised. Current methods to monitor for *Cryptosporidium* oocysts in water are microscopy-based USEPA Methods 1622 and 1623. These methods assess total levels of oocysts in source waters, but do not determine oocyst viability or genotype. Recently, propidium monoazide (PMA) has been used in conjunction with molecular diagnostic tools to identify species and assess the viability of bacteria. The goal of this study was the development of a *Cryptosporidium* propidium monoazide-polymerase chain reaction (CryptoPMA-PCR) assay that includes PMA treatment prior to PCR analysis in order to prevent the amplification of DNA from dead oocysts. The results demonstrated that PMA penetrates only dead oocysts and blocks amplification of their DNA. The CryptoPMA-PCR assay can also specifically detect live oocysts within a mixed population of live and dead oocysts. More importantly, live oocysts and not dead oocysts were detected in raw waste or surface water samples spiked with *Cryptosporidium* oocysts. This proof of concept study is the first to demonstrate the use of PMA for pre-PCR treatment of *Cryptosporidium* oocysts. The CryptoPMA-PCR assay is an attractive approach to specifically detect and genotype viable *Cryptosporidium* oocysts in the water, which is critical for human health risk assessment.

INTRODUCTION

Cryptosporidium is a protozoan parasite infecting many animals and humans. Infection occurs following ingestion of oocyst-contaminated food, drinking or recreational waters. It can also be transmitted from human to human or animal to human (13). Typically, it causes a self-limiting diarrheal disease in immunocompetent individuals, but can become severe and even lead to death in the immunocompromised (e.g., AIDS patients, elderly, and infants) (51). This parasite has been detected in many drinking water sources and is considered an important waterborne contaminant (52). The *C. hominis*-linked outbreak in Milwaukee, WI, USA in 1993, which involved over 400,000 infections and at least 54 deaths, is a clear example of a cryptosporidiosis outbreak due to oocyst contaminated drinking water (24, 27). There are currently twenty valid species and over forty genotypes of this parasite. *C. canis, C. felis, C. hominis*, *C. meleagridis, C. muris, C. parvum, C. suis,* and the cervine, chipmunk, rabbit, and horse genotypes have been shown to cause disease in humans (13, 14, 36, 38). Of these, *C. parvum* and *C. hominis* cause over 95% of the reported cases of human cryptosporidiosis (52).

To reduce health risks posed by this parasite, the USEPA promulgated Long Term 2 Enhanced Surface Water Treatment Rule (LT2). Under this rule, drinking water utilities that use surface water or ground water under the direct influence of a surface water as their primary drinking water source are required to monitor their source waters for *Cryptosporidium* oocysts using USEPA Methods 1622 or 1623 (43-45). These microscopic-based methods produce total counts of live and dead *Cryptosporidium* oocysts in water samples without distinguishing species or genotypes that can infect humans from those that cannot. Results are then used to determine the need for additional water treatment in order to reduce the public health burden of waterborne

66 cryptosporidiosis. These methods can overestimate the concentration of oocysts infectious for
67 humans in water supplies and health risks posed by waterborne exposures to this parasite.
68 Many genotyping methods have been used to identify anthroponotic and zoonotic
69 *Cryptosporidium* species in water supplies (52). These include the use of PCR-restriction
70 fragment length polymorphism (PCR-RFLP), single-strand conformational polymorphism
71 (SSCP), PCR-randomly amplified polymorphic DNA (PCR-RAPD) (15, 54), and PCR-DNA
72 sequencing (54). Despite being instrumental in illustrating the immense diversity of
73 *Cryptosporidium* spp. present in the environment, these methods cannot distinguish viable from
74 non-viable oocysts.
75 Alternative approaches that do distinguish viable from non-viable oocysts have been
76 developed. For example, rodent models of infection are used to measure the infectivity of this
77 parasite (3, 23, 29) but this approach is time consuming, labor intensive, and expensive. In
78 addition, it can only determine infectivity of a select number of *Cryptosporidium* species. More
79 rapid alternative techniques that do not use animal models include cell culture (37, 39) and i*n*
80 *vitro* excystation assays (23). The cell culture assay, however, is also limited in its range. It can
81 only be used to detect viable *C. andersoni*, *C. hominis C. parvum, C. meleagridis* and *C. muris*
82 oocysts but not viable oocysts of other *Cryptosporidium* species (1, 8, 19). *In vitro* excystation
83 assays can overestimate oocyst viability, particularly if oocysts have been treated with various
84 disinfectants (6, 7, 16). Other techniques use vital dye staining approaches like 4',6-diamidino-2-
85 phenylindole (DAPI), propidium iodide (PI) and SYTO®-9. These approaches can also be
86 unreliable and can overestimate the total number of viable oocysts in water samples, especially if
87 the water was chemically disinfected (6, 7, 9, 21). Other more recent approaches utilize
88 molecular-based techniques to assess oocyst viability. Fluorescence *in situ* hybridization (FISH)

89 (5, 10, 20, 26, 40, 47), Nucleic Acid Sequence Based Amplification (NASBA) (4), reverse-
90 transcriptase PCR (RT-PCR) (17, 22, 50), and an integrated cell culture/PCR assay (12, 25) all
91 show promise in providing a rapid and relatively inexpensive viability assay for
92 *Cryptosporidium*. However, it still remains to be determined if these methods can detect all
93 *Cryptosporidium* species and genotypes found in the environment.
94 It has been demonstrated recently that propidium monoazide (PMA) or ethidium
95 monoazide (EMA) treatment, in conjunction with molecular methods, can be used for selective
96 detection and genotyping of viable bacteria (30, 34) and fungi (48). PMA and EMA are new
97 photoactive vital dyes that can preferentially penetrate dead cells, or cells with damaged or
98 permeabilized cell membranes, but not viable cells with intact cell membranes. Once inside the
99 cell, these molecules then intercalate into the DNA and become covalently bound to DNA upon
100 exposure to bright white light. This photoactivation process results in the formation of a stable
101 DNA-PMA/EMA complex that renders the DNA inaccessible for PCR amplification (31, 33).
102 However, Nocker and colleagues have reported that EMA can also penetrate live bacteria and
103 suggested that PMA is more effective at discriminating live from dead bacteria (31). In this
104 paper, we developed and evaluated the use of PMA to selectively block PCR amplification of
105 DNA from dead *Cryptosporidium* oocysts. Following PMA treatment, the *SSU rRNA* and *hsp 70*
106 genes were PCR amplified and sequenced to determine the genotypes of these oocysts. This
107 "CryptoPMA-PCR" approach was then successfully applied to selectively detect viable oocysts
108 in a mixed suspensions of viable and dead oocysts that were spiked into various environmental
109 matrices. This new Crypto-PMA-PCR assay, which allows genotyping and viability
110 determination, has a potential to substantially improve the data on waterborne exposures to
111 *Cryptosporidium* and enhance the validity of human risk assessment.

MATERIALS AND METHODS

***Cryptosporidium* oocysts.** *C. parvum* oocysts (Iowa isolate, Harley Moon strain) were purchased from Waterborne, Inc., and propagated in C57BL/6 mice at the U.S. EPA facility in Cincinnati, OH as previously described (28). Oocysts were purified from the feces by sieving, step sucrose gradients, and cesium chloride purification (3). Purified oocysts were then suspended in reagent grade water containing 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Gaithersburg, MD) and stored at 4 ^{o}C. *C. muris* oocysts (RN66), originally obtained from Dr. U. Iseki, (Osaka University, Medical School, Osaka, Japan), were propagated in CF-1 female mice, purified from the feces by sieving and sucrose flotation , and stored at 4 ^{o}C in 0.01% Tween-20 containing 100 U penicillin/mL and 100 μg streptomycin/mL as described by Miller, et. al.(28). For all experiments, oocysts were used within three months of isolation with the exception of intentionally aged oocysts that were kept at ambient temperature (20-22 °C) for 14 months in the presence of Tween-20 and antibiotics as described above.

Heat inactivation. 2 ml Costar natural clear microcentrifuge tubes (Corning Inc., Corning, NY) with oocyst suspended in PBS were heat treated in a water bath at 70 ºC for 30 min. Oocyst infectivity was determined by cell culture as described below.

PMA treatment of oocysts. Propidium monoazide (PMA) (Biotium, Inc., Hayward, California) was received lyophilized and reconstituted with 20 % DMSO to a stock concentration of 20 mM and aliquoted in 5 μL volume and stored at 4 °C for no longer than 2 months. The PMA stock solution was diluted with PBS to final concentrations ranging from 5 μM to 150 μM depending

on the experiment. Oocysts were incubated with PMA for 5-30 min in a 2 ml Costar natural clear microcentrifuge tube (Corning Inc.) with vortexing for 10 sec every 5 min. Samples were then exposed to a Cosmobeam 800-W halogen light source (Cosmolight, Rome, Italy) at a distance of 20 cm while horizontal in ice for photo-induced cross-linking of PMA to oocyst DNA. Light exposure time ranged between 1 to 5 min depending on the experiment. Exposures were in 30 sec increments with 1 min rest in between exposures to avoid damaging the oocysts.

Flow sorting of *C. parvum* samples. A Fluorescence Activated Cell Sorter (FACS VantageSE, Beckton Dickinson, Palo Alto, CA) equipped with CloneCyt software was used for enumerating oocysts (49). Oocysts were gated by forward and side scatter (FSC and SSC) with FSC and SSC measured linearly with a gain of 1, SSC set at 399 V, and FSC threshold set at 32 V, reflecting their size and shape. This gate was used for live and heat-killed oocysts. Sorted oocysts between 1×10^4 and 1×10^6 were collected in sterile 1.5 mL microfuge tubes containing 0.5 mL of sterile PBS.

Infectious foci detection and *in vitro* cell culture of *Cryptosporidium* oocysts. Human ileocecal adenocarcinoma cells (HCT-8) were purchased from the American Type Cell Culture (ATCC CCL 244, Manasas, VA) and maintained as previously described (11). Briefly, HCT-8 cells were grown as monolayers in 75-cm^2 cell culture flasks in maintenance media consisting of RPMI 1640 medium (Gibco, Gaithersburg, MD), 5% fetal bovine serum (Hyclone, Logan, UT), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco) at 37ºC with 5% CO_2. For infectivity experiments, cells were grown on 25 mm coverslips in six-well Costar tissue culture plates

157 (Corning, Inc.) to 80-85% confluency. Cells were then washed three times with PBS and fresh
158 maintenance media was added prior to the addition of oocysts.
159 Live or heat-killed *C. parvum* oocysts were pretreated with 5% sodium hypochlorite
160 (Sigma-Aldrich, St. Louis, MO) for 5 min on ice, followed by three washes with PBS prior to
161 inoculation. 1 x 10^6 *C. parvum* oocysts were then added to the coverslips as previously
162 published (39). At 24 and 72 h post-inoculation, coverslips containing infected cells were
163 washed 3 times in ice-cold PBS and fixed with 4% paraformaldehyde for 10 min. Cells were
164 then permeabilized with 0.25% Triton X-100 (Sigma) for 10 min, followed by a blocking step
165 using fresh 1 % BSA in PBS. *Vicia villosa* lectin (VVL) (Vector Laboratories, Burlingame, CA)
166 was used to detect parasites as previously described (18).
167
168 **Genomic DNA extraction, PCR amplification and sequencing.** Genomic DNA from 1 x 10^4-
169 1 x 10^5 PMA treated and untreated live and heat-killed oocysts were isolated using the
170 MasterPure DNA Purification Kit in accordance with manufacturer's protocol (Epicentre
171 Biotechnologies, Madison, Wisconsin). Briefly, oocysts resuspended in tissue and cell lysis
172 solution were subjected to five freeze/thaw cycles followed by an overnight Proteinase K
173 digestion at 55 ºC. Samples were then treated with RNase and protein precipitation reagent.
174 After the removal of cell debris by centrifugation, genomic DNA was precipitated with
175 isopropanol, followed by two 75% ethanol wash steps, and then resuspended in 35 µL of
176 nuclease-free water (Invitrogen, Grand Island, NY).
177 Primers that amplified a 346-bp length of the *C. parvum hsp70* gene, as described by Di
178 Giovanni et. al. (12), were used to determine presence or absence of amplifiable DNA following
179 PMA treatment. Sequences were as follows: forward primer, 5'-TCC TCT GCC GTA CAG

GAT CTC TTA-3' and reverse primer, 5'-TGC TGC TCT TAC CAG TAC TCT TAT CA-3'. PCR conditions consisted of an initial denaturation at 95 °C for 10 min followed by 30 cycles of 95 °C for 30 sec, 60 °C for 1 min, 72 °C for 2 min with a final extension step at 72 °C for 10 min.

An 834 bp portion of *C. parvum SSU rRNA* or 824 bp portion of *C. muris SSU rRNA* were also amplified to determine presence or absence of amplifiable DNA following PMA treatment using the following previously published sequences: forward primer 5'-GGA AGG GTT GTA TTT ATT AGA TAA AG-3' and reverse primer 5'-AAG GAG TAA GGA ACA ACC TCC A-3' (53). PCR conditions for the *SSU rRNA* reactions were as follows: an initial denaturation step at 95 °C for 4 min followed by 30 cycles at 94 °C for 45 sec, 58 °C for 45 sec and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. All 40 μL PCR reactions contained 2.5 U AmpliTaq Gold, 4 mM $MgCl_2$, 1X PCR buffer 2 (Applied Biosystems),1 μM of each primer and 2 μL of genomic DNA. 1uL of the PCR product was visualized using the DNA 1000 kit for the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA).

For sequence analysis, PCR fragments were purified with the QIAquick PCR Purification Kit (QIAGEN, Valencia, California) and sent to Cincinnati Children's Hospital Medical Center DNA Core facility for sequencing. The Lasergene 7 software package was used to analyze DNA sequences.

Analysis of *Cryptosporidium* oocysts by immunofluorescence. Oocysts were stained using Crypt-a-Glo fluorescent antibody staining kit in accordance to manufacturer's instruction (Waterborne, New Orleans, LA). Microscopic examinations were performed with a Zeiss Axiphot2 epifluorescence microscope (Carl Zeiss) equipped with bright field, DIC, and

203 epifluorescence optics. The oocysts stained with FITC-conjugated antibody were observed using
204 a FITC filter set, while oocysts with PMA-labeled DNA were observed using a
205 Rhodamine/Texas-Red filter set. Photomicrographs were taken using AxioVision (Carl Zeiss)
206 and analyzed with Adobe Photoshop (Adobe, San Jose, CA).
207
208 **Detection of live and dead oocysts in a mixed sample.** FACS sorted oocysts of live or heat-
209 killed *C. parvum* or *C. muris* were mixed at defined ratios of 0:100 (0:10,000 oocysts), 1:99
210 (100:9,900 oocysts), 10:90 (1,000:9,000 oocysts), 50:50 (5,000:5,000 oocysts), 90:10
211 (9,000:1,000 oocysts), 99:1 (9,900:100 oocysts), or 100:0 (10,000:0 oocysts), respectively,
212 treated with PMA and then exposed to an 800W halogen light. Total genomic DNA from the
213 oocyst mixture was then extracted and analyzed using a single round of PCR amplification of the
214 *SSU rRNA* and *hsp70* gene followed by sequencing as described previously (53).
215
216 **Detection of viable oocysts in environmental samples.** 20 L water samples were collected in
217 carboys from the Ohio River. Water turbidity was 17 Nephelometric Turbidity Units (NTU).
218 Samples were filtered in the laboratory using a portable gear pump and Filta-Max compressed
219 foam filters (IDEXX Laboratories, Westbrook, Maine). Filters were eluted into 500 mL conical
220 centrifuge tubes using a Filta-Max *xpress* automatic Pressure Elution Station in accordance with
221 the manufacturer's instructions and Method 1623 (in 2007 USEPA accepted the use of Filta-
222 Max^{TM} xpress as a sample processing option for Methods 1622 and 1623) (44). The eluate was
223 centrifuged at 3,000 × *g* for 15 min. The supernatant was then carefully aspirated and discarded.
224 The centrifuged pellet (2 mL volume) was resuspended in 50 mL PBS and divided into five 10

mL aliquots containing 0.4 mL packed pellet each. These aliquots were dispensed into 12 mL flat-sided Leighton tubes.

For raw wastewater samples, 50 mL of raw wastewater was divided into five 10 mL aliquots each containing 58 mg of solids and transferred into flat-sided 12 mL Leighton tubes.

1×10^5 live or heat-killed *C. parvum* oocysts were then spiked into each tube containing resuspended Ohio River packed pellet or raw wastewater. Oocysts were separated from the sample matrix using a Dynabeads ImmunoMagnetic Separation (IMS) kit, which employs magnetic beads covalently coupled to anti-*Cryptosporidium* antibody (Invitrogen Dynal AS, Oslo, Norway) in accordance with the manufacturer's guidelines and method 1622. After separation from the sample matrix, the oocyst-bead complexes were resuspended in 200 µL PBS and treated with PMA as described above.

RESULTS

Microscopic analysis of live and dead *C. parvum* oocysts treated with PMA

Live or heat-killed oocysts, which were incubated with 25 μM PMA for 10 min, were stained with FITC-conjugated anti-*Cryptosporidium* antibody (Crypt-a-Glo) and analyzed using fluorescence microscopy (Fig. 1). Following PMA treatment, live oocysts remained impermeable to PMA and did not stain red (Fig. 1B), whereas heat-killed oocysts stained bright red (Fig. 1E). Figure 1C and F are overlain images of Crypt-a-Glo stained oocysts (Fig. 1A and D) and PMA (Fig. 1 B and E). Dead oocysts (Fig. 1F) that are both Crypt-a-Glo and PMA positive appear yellow. Further analysis revealed that less than 10% of oocysts which were not treated with heat were positive for PMA, while greater than 95% of heat-killed oocysts were PMA positive. The observed PMA staining of some untreated oocysts seen in Fig. 1B was likely due to the natural attrition of oocysts that occurs over time and/or oocysts killed during the purification process. To confirm that PMA negative and PMA positive oocysts were indeed live or dead, respectively, we performed a cell culture infectivity analysis of these oocysts using HCT-8 cells. PMA negative oocysts infected HCT-8 cells easily as indicated by the presence of multiple infectious foci at 24 h post-infection which increased in numbers at 72 h post-infection. In contrast, no infectious foci were detected at both time points when cells were inoculated with heat-killed oocysts that previously stained positive for PMA (data not shown).

CryptoPMA-PCR assay to detect live but not dead *Cryptosporidium* oocysts

To determine optimal PMA concentrations for live/dead oocyst discrimination using PCR, oocysts were stained with PMA concentrations from 0.5 to 150 μM. 1 x 10^4 live or heat-

killed oocysts were incubated with PMA for 10 min followed by light exposure for 2 min. This was followed by DNA extraction and PCR amplification of the *SSU rRNA* gene (Fig. 2A). Our results revealed that PMA concentration as high as 150 μM had no noticeable inhibitory effects on the detection of live oocysts by PCR as indicated by the presence of a PCR product of the expected size with similar band intensity as compared with PCR products detected from live untreated oocysts (Fig. 2A, Lanes 2 and 3-6). By contrast, PMA treatment as low as 0.5 μM resulted in a marked reduction of detectable PCR products as compared to heat-killed oocysts which were not treated with PMA (Fig. 2A, Lane 7 and 8). More remarkable is the complete loss of amplicons from dead oocysts incubated with 5, 50, or 150 μM PMA (Fig. 2A, Lanes 9-11).

To determine if this CryptoPMA-PCR assay can also work on another *Cryptosporidium* gene locus used for genotyping, the *hsp70* gene was also tested. As shown in Figure 2B, the *hsp70* gene was easily amplified from live untreated *C. parvum* oocysts and was also detected with no marked inhibition of PCR amplification in live *C. parvum* oocysts treated with PMA (Fig. 2B, Lanes 2-6). PCR products were also detected from untreated dead oocysts, albeit with slightly reduced band intensity (Fig. 2B Lane 7). In contrast, treatment with as little as 0.5 μM PMA (Fig. 2B, Lane 8) resulted in an almost complete loss of the *hsp70* PCR product. Treatment with higher PMA concentrations up to 150 μM resulted in undetectable levels of PCR products (Fig. 2B Lanes 9-11). These results demonstrate that *SSU rRNA* and *hsp70* genes, two gene loci commonly used for genotyping *Cryptosporidium* spp. (15), are useful targets for this CryptoPMA-PCR detection assay. More importantly, pre-treatment of *Cryptosporidium* oocysts with PMA resulted in the selective PCR amplification of the *SSU rRNA* or *hsp70* genes from live oocysts and the concomitant inhibition of PCR amplification of these genes from dead oocysts.

Optimizing PMA incubation times and photoactivation conditions for the CryptoPMA-PCR assay

To optimize PMA incubation time, *C. parvum* oocysts were incubated on ice for 5 min or 30 min with 150 μM PMA followed by a 2 min exposure to bright light, a photoactivation step required to covalently bind PMA to DNA (Fig. 3) (31, 33). Incubation of live oocysts with PMA for 5 min (Fig. 3, Lanes 3 and 12) or 30 min (Fig. 3, Lanes 4 and 13) followed by bright light exposure did not have an appreciable inhibitory effect on amplifying the *SSU rRNA* or *hsp70* gene. Conversely, the PMA molecule readily penetrated and intercalated into DNA of heat-killed oocysts as seen by fluorescence microscopy (Fig. 1E) resulting in the loss of detectable *SSU rRNA* or *hsp70* PCR amplicons at 5 min (Fig. 3, Lanes 6 and 15) or 30 min (Fig. 3, Lanes 7 and 16) incubation times. These results revealed that incubation of oocysts for at least 5 min allowed sufficient time for PMA to cross several complex barriers (oocyst wall, sporozoite cell and nuclear membranes) and intercalate within the DNA structure to prevent amplification of genomic DNA from dead oocysts. Incubation of live oocysts with PMA for up to 30 min did not prevent amplification of their DNA nor did it result in any observable reduction in PCR products detected as compared with live untreated oocysts (Fig. 3, Lanes 2-4 and 11-13).

To minimize excessive heating of the oocysts, heat-induced oocyst excystation, or light induced DNA damage during the PMA photoactivation step, light exposure times between 1 min to 5 min were evaluated. Figure 4 illustrates that a 1 min exposure to bright light (800 W) was sufficient to photoactivate PMA and prevent amplification of both target genes from heat-killed oocysts (Fig. 4 , Lanes 9 and 23) with no observable reduction of PCR products detected from live oocysts (Fig. 4, Lanes 3 and 17). Exposure times up to 4 min also had no detectable

306 inhibitory effects on amplifying the *SSU rRNA* or the *hsp70* gene in live oocyst samples;
307 however, a 5 min light exposure showed a slight reduction in detectable *SSU rRNA* but not the
308 *hsp70* amplicons from live oocysts (Fig. 4, Lane 7 vs. 21). Based on these experiments, oocysts
309 were incubated with 150 μM PMA for 5 min followed by a 2 min light exposure time in all the
310 experiments described below.
311
312 **Molecular genotyping of live *Cryptosporidium* oocysts in a mixture of live and dead oocysts**
313 **of different species**
314 To determine if this CryptoPMA-PCR assay can be applied for the detection and
315 genotyping of live *Cryptosporidium* oocysts in a mixed population containing different
316 *Cryptosporidium* species, live or heat-killed *C. muris* oocysts were mixed with live or heat-killed
317 *C. parvum* at defined ratios (Table 1). These samples were then treated with PMA followed by
318 PCR reactions amplifying the *SSU rRNA* region. Detected amplicons were then sequenced to
319 identify the species. Results in Table 1 demonstrate that in 2 of 3 experiments performed, live *C.*
320 *parvum* oocysts were detected when mixed with dead *C. muris* at a 10:90 *C. parvum: C. muris*
321 ratio. Additional experiments also revealed that only *C. parvum* DNA was detected in all three
322 experiments when live *C. parvum* and dead *C. muris* oocysts were mixed at a 50:50 ratio. No *C.*
323 *muris SSU rRNA* sequences were detected when dead *C. muris* oocysts were analyzed.
324 Similarly, when ratios of 50:50, or 10:90 live *C. muris* to heat-killed *C. parvum* oocysts,
325 respectively, were analyzed, only the *C. muris SSU rRNA* sequence was detected in all six
326 experiments performed. The ability of this CryptoPMA-PCR assay to detect 100 live *C. muris*
327 oocysts in the presence of 9,900 heat-killed *C. parvum* oocysts (1:99 ratio) in one of three
328 experiments performed is also notable.

329

330 **Application of the CryptoPMA-PCR assay for the detection of live oocysts in**

331 **environmental matrices**

332 To determine if the CryptoPMA-PCR assay is suitable for the detection and genotyping
333 of live oocysts in complex matrices, samples of untreated water from the Ohio River and raw
334 wastewater samples from a municipal sewage treatment plant were each spiked with live or dead
335 *C. parvum* oocysts. These matrices were chosen because they contain debris and dissolved
336 compounds (Fig. 5A), which may not be completely separated from oocysts by the IMS
337 procedure and can physically block entry of PMA into oocysts, sequester PMA away from the
338 oocysts and/or mask light exposure during the photoactivation step. There was nearly a
339 complete loss of the *SSU rRNA* PCR product from heat-killed PMA treated oocysts spiked into
340 the raw surface water (Fig. 5B Lane 5). Similarly, a marked reduction in the amount of
341 amplicons was observed when analyzing the PMA treated heat-killed oocysts, which were spiked
342 in the raw wastewater (Fig. 5B, lane 10). The presence of a faint band in the raw wastewater
343 sample may be a result of the presence of excess free DNA in the sample binding PMA and/or
344 masking effects of debris present in the matrix preventing the complete removal and/or inhibition
345 of PCR amplification of spiked dead oocysts.

346

347 **Using CryptoPMA-PCR assay to determine viability of *Cryptosporidium* oocysts stored at**

348 **ambient temperatures**

349 Previous studies by Jenkins and others have shown that long term storage of oocysts at
350 ambient temperatures resulted in the loss of oocyst infectivity due to natural oocyst attrition as a
351 consequence of time and temperature (22). To determine if the CryptoPMA-PCR assay can be

352 used to discriminate viable *Cryptosporidium* oocysts from oocysts that lost viability as a result of
353 long-term storage at room temperature, 1 x 10^4 live, heat-killed, or aged (stored at 20-22 °C for
354 14 months) oocysts were analyzed as described above. PCR amplification of the *SSU rRNA* gene
355 from DNA equivalent to 100 live or heat-killed oocysts in the absence of PMA pre-treatment
356 resulted in a distinct band (Fig 6A, lanes 2 and 4). No difference in band intensity was observed
357 with the addition of PMA in live oocysts, while no PCR product was observed in heat-killed
358 oocysts treated with PMA (Fig 6A, lanes 3 and 5).

359 Furthermore, when the *SSU rRNA* region was amplified from DNA equivalent to 10
360 oocysts, amplicons were still detected in both live oocysts samples with or without PMA or heat-
361 killed oocysts in the absence of PMA (Fig. 6B, lanes 9-11). When the *SSU rRNA* gene was PCR
362 amplified from 100 aged oocysts, a distinct, albeit faint band, was detected in the absence of
363 PMA, which was completely abolished upon the addition of PMA (Fig 6A, lanes 6 and 7
364 respectively). No amplicons were detected from ten PMA-treated heat-killed oocysts (Fig. 6B,
365 lane 12), or ten aged oocysts in the presence or absence of PMA (Fig. 6B, lanes 13 and 14,
366 respectively).

367 These results demonstrate that the CryptoPMA-PCR assay is effective at preventing the
368 amplification of dead oocysts in a sample that has been kept at ambient temperatures for 14
369 months, conditions that oocysts can be exposed to in the natural environment. Moreover, these
370 results also demonstrate the single round of PCR used for the CryptoPMA-PCR is able to detect
371 DNA equivalent to 10 live oocysts.

DISCUSSION

Current methods for determining viability employ cell culture or rodent models of infection (37). However, cell culture-based and animal bioassay approaches have inherent limitations. For example, it is not known if all species and genotypes of *Cryptosporidium* can grow in cell culture (2). Little is known about the susceptibility of rodents to infection with many species and genotypes of this parasite that can contaminate water supplies (42). Vital dyes like PI, DAPI and SYTO®-9 have been used as rapid alternatives to animal bioassays or cell culture assays in determining viability of oocysts, but results from these approaches can be variable and offer limited genotyping capabilities (6, 7, 9, 21, 29). PMA and EMA represent a new generation of vital dyes that contain a photoreactive azide group that can covalently bind to nucleic acids following photolysis. And unlike PI, which can diffuse in and out of oocysts (6, 7), photoactivated PMA or EMA forms a stable complex with nucleic acids that does not diffuse out of oocysts, (31). Studies have shown however, that EMA can overestimate the amount of viable bacteria (31). Thus this study used PMA as a pre-PCR treatment for the CryptoPMA-PCR assay. An advantage of the Crypto-PMA-PCR assay is that it is a rapid approach that does not involve cell culture. In addition, this approach allows for downstream genotyping following PMA treatment.

This proof of concept study is the first to demonstrate that heat-killed and oocysts that were inactivated by long-term storage at room temperature were permeable to PMA, whereas live oocysts were impermeable to it. Since PMA can cross multiple parasite membranes to intercalate and covalently bind to *Cryptosporidium* genomic DNA it can be used as a pre-PCR treatment that renders dead oocysts undetectable by PCR analysis. For example, in the absence of a PMA labeling step, the *SSU rRNA* or *hsp70* genes from live, heat-killed, or inactivated by

long-term storage *Cryptosporidium* oocysts were both amplified, whereas these genes were only detected from live *Cryptosporidium* oocysts when the PMA labeling step was performed. As illustrated in Figure 6 and Table 1, this method was able to detect relatively low numbers of live oocysts (10-100 oocysts) in a pure preparation as well as in a mixture with at least 9,900 dead oocysts. Conversely, these target genes were not detected from oocysts exposed to room temperature for fourteen months or heat-killed oocysts at doses up to 10,000 oocysts further demonstrating the efficiency of PMA to prevent amplification of DNA from dead *Cryptosporidium* oocysts. This study builds upon previous findings by Nocker and others on the use of PMA to selectively detect and genotype viable bacteria and extends the application of PMA treatment to a more complex parasite (32). In this study, a single round PCR was effective at detecting DNA from as few as 10 live oocysts (Fig. 6). We anticipate that combining the PMA staining step with a more sensitive genotyping approach, such as nested PCR, will enable the detection of a single live oocyst in environmental samples (15, 53). However, great care should be taken with nested PCR since this approach can also increase a risk of false positive results, particularly when environmental samples are analyzed. Additional experiments are also warranted to further evaluate the utility of PMA for the differentiation of live and dead oocysts using other molecular-based detection methods not described here.

The addition of PMA was effective at preventing the amplification of DNA from oocysts that were killed by exposure to heat or long-term storage at room temperature suspended in distilled water (Fig. 6). However, a faint band was detected in oocyst-free sewage samples that were spiked with heat-killed oocysts. This result could be due to the presence of high levels of indigenous extracellular DNA in waste water matrix, which can sequester free PMA molecules away from oocysts, thereby reducing the effectiveness of PMA in inhibiting amplification of

418 DNA from dead oocysts. Two recent studies by Varma and others also reported similar findings
419 when using PMA to detect bacteria in wastewater indicating the difficulty in using PMA on
420 wastewater matrices (46). Alternatively, photoactivation of PMA could have been prevented
421 through a "masking" effect by debris and contaminants that may have carried over during the
422 IMS step. It is also possible that the beads themselves can interfere with the photoactivation
423 process. However, PCR amplification of the *SSU rRNA* gene from live oocysts treated with
424 PMA and photoactivated in the presence of at least 10^5 magnetic beads had no effects on the
425 levels of PCR amplicons detected (data not shown). To minimize the effects of indigenous
426 DNA, suspended solids, and dead microorganisms on PMA staining of dead *Cryptosporidium*
427 oocysts in environmental samples, adding excess amounts of PMA as well as increasing light
428 exposure should be considered as potential strategies. Nevertheless, this study demonstrated that
429 the CryptoPMA-PCR assay was an improvement to the specificity of current PCR techniques
430 used to detect live *Cryptosporidium* oocysts seeded in turbid waters, like raw surface water
431 samples (Figure 5B).
432 The mode of action of PMA is still not completely understood. Previous studies by
433 Nocker and colleagues demonstrated that once PMA intercalates into and becomes covalently
434 bound to DNA of bacteria, the PMA-DNA complex becomes insoluble and is subsequently
435 removed during DNA extraction procedures (31). These scientists also hypothesized that bound
436 PMA may affect Taq polymerase-based PCR amplification of the target DNA (32). Studies
437 using EMA to differentiate live from dead *L. monocytogenes* bacteria have suggested that the
438 duration of light exposure of DNA in the presence of EMA can inhibit PCR amplification of
439 target genes (41). Whether PMA has similar effects as EMA remains to be determined. Despite
440 our limited knowledge of its mode of action, PMA treatment is very effective at preventing the

amplification of the *hsp70* and *SSU rRNA* genes from heat-killed *Cryptosporidium* oocysts. Interestingly, during the optimization of the photoactivation process, we observed that exposure to light for at least 5 min resulted in a marked decrease in the signal intensity of the *SSU rRNA* amplicon, but not of the *hsp 70* gene. This may be attributed to light-induced DNA strand breaks leading to decreased signal intensity for the *SSU rRNA* amplicon. These results were also seen in previous studies done in other eukaryotic cells (35). The *SSU rRNA* amplicon is longer that the *hsp 70* amplicons by nearly 500 bp. The relatively small size of the *hsp 70* amplicon (346 bp) may explain why it might have a lower probability of light induced strand breaks. Since 2 min of light exposure did not result in any appreciable decrease in signal intensity for either *SSU rRNA* or *hsp 70* amplicons from live *Cryptosporidium* oocysts, we chose this duration of light exposure for all further experiments performed in this study. Nonetheless, great care should also be taken when optimizing this approach to detect other microorganisms.

Here, we have developed the CryptoPMA-PCR technique that is very effective at specifically detecting and genotyping live oocysts seeded in distilled water samples and also has a potential to specifically detect viable oocysts in environmental water samples. Unlike RT-PCR, which is labor intensive and requires advanced technical skills to manipulate messenger RNA (a very labile molecule that easily degrades), the CryptoPMA-PCR assay uses DNA as its template, which makes this technique more versatile and user-friendly. The CryptoPMA-PCR can be modified to use other types of molecular genotyping techniques, such as nested PCR, single strand conformational polymorphisms, or multi-locus sequence typing. The use of quantitative real-time PCR with PMA pre-treatment may also enable quantitation of viable pathogenic *Cryptosporidium* oocysts in environmental samples.

463 Future research efforts should also focus on determining sensitivity and versatility of
464 PMA to distinguish live from dead oocysts that have been treated with different methods of
465 disinfection (e.g. UV, chlorine, ozone, and natural sunlight). Previous studies have demonstrated
466 that real-time PCR plus PMA treatment could not be used to differentiate UV-inactivated
467 bacteria from viable ones suggesting that PMA pre-treatment may also be limited to preventing
468 PCR amplification of DNA from oocysts that were treated with heat or chemicals (e.g., chlorine,
469 ozone, aldehydes, and alcohols) that make the oocyst wall permeable for PMA (33). More
470 importantly, standardization and inter-laboratory performance comparison of this assay, using
471 different water matrices spiked with low numbers of oocyst typically found in environmental
472 samples, must be performed in order to evaluate the practical applicability of this approach for
473 water quality monitoring.
474 This CryptoPMA-PCR approach is a novel tool that combines genotyping and viability
475 determination. After further refinement and validation, this method may be used in source
476 tracking studies or for generating improved data on waterborne exposures to *Cryptosporidium*
477 and assessing health risks posed by this parasite.

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AUTHOR CONTRIBUTIONS

CCB and ENV conceived and designed the experiments. CCB, SMG, MWW, EAV, and AIE performed the experiments. CCB and ENV analyzed the data. CCB, ENV, SMG, MWW, EAV, and AIE contributed reagents/materials/analysis tools. CCB and ENV wrote the paper.

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663 **FIGURE LEGENDS**

664

665 **Figure 1**. Microscopic analyses distinguishing live from dead *C. parvum* oocysts using PMA.
666 (A-C) Live or (D-F) heat-killed (70 ºC, 20 min) *C. parvum* oocysts treated with PMA and stained
667 with Crypt-a-Glo. Crypt-a-Glo labeled oocysts (green) are shown in A and D, PMA staining
668 (red) of the same oocysts is shown in B and E, while C and F represent overlain images with
669 Grypt-a-Glo and PMA (yellow) staining. Bar = 10 μm.

670

671 **Figure 2**. Effects of different PMA concentrations on detecting live and heat-killed *C. parvum*
672 oocysts. Live or heat-killed *C. parvum* oocysts were incubated with 0.5, 5, 50 or 150 μM PMA
673 for 10 min followed by a 2 min light exposure. This was followed by the extraction of genomic
674 DNA and PCR amplification with primers targeting the (A) *SSU rRNA* or (B) *hsp 70* genes.
675 PCR products were analyzed using an Agilent Bioanalyzer 2100. M: Marker; - : No PMA
676 control; Δ: 70 ºC, 30 min treated oocysts; C: PCR control that lacks template.

677

678 **Figure 3.** Effects of PMA incubation time on detecting live and heat-killed *C. parvum* oocysts.
679 Live or heat-killed *C. parvum* oocysts incubated with 150 μM PMA for either 5 or 30 min and
680 then exposed to light for 2 min. Genomic DNA was purified as described in the Materials and
681 Methods. Amplification of a portion of the *hsp70* (Lanes 2-9) or *SSU rRNA* (Lanes 11-18) genes
682 was performed using PCR. Gel electrophoresis analysis was performed using an Agilent
683 Bioanalyzer 2100. M: Marker, - : No PMA control, Δ: 70 ºC treated oocysts, C: PCR control
684 that lacks template.

685

686 **Figure 4.** Effects of the duration of exposure to light after PMA treatment on detecting live and
687 heat-killed *C. parvum* oocysts. Live or heat-killed *C. parvum* oocysts were incubated with 150
688 μM PMA for 5 min and exposed to light for 1, 2, 3, 4, or 5 min. Genomic DNA was purified the
689 *hsp70* (Lanes 1-14) or *SSU rRNA* (Lanes 15-28) genes were amplified by PCR. Gel
690 electrophoresis analysis was performed using an Agilent Bioanalyzer 2100. M: Marker, - : No
691 PMA control, Δ: 70 ºC treated oocysts, C: PCR control that lacks template.
692
693 **Figure 5**. Detection of *C. parvum* oocysts in Ohio River and raw wastewater samples using
694 CryptoPMA-PCR. (A) Environmental samples spiked with oocysts prior to immunomagnetic
695 separation, PMA treatment and PCR: resuspended 0.4 mL pellet from raw surface water
696 concentrate (1) and raw wastewater (2)**.** (B) *SSU rRNA* PCR amplicons from live and dead
697 oocysts spiked in raw surface water or raw wastewater. Amplification of a portion of the *SSU*
698 *rRNA* gene was performed using PCR (Lanes 2-10). Gel electrophoresis analysis was performed
699 using an Agilent Bioanalyzer 2100. M: Marker, - : No PMA, +: PMA added, Δ: 70 ºC treated
700 oocysts, B: Blank sample that did not receive oocyst spike. Data represent one of two
701 independent experiments performed.
702
703 **Figure 6.** Differentiation of live *C. parvum* oocysts from heat-killed oocysts (Δ) and oocysts
704 inactivated by long-term storage at room temperature (Aged) using CryptoPMA-PCR. Live
705 (lanes 2, 3, 9 and 10), heat-killed (lanes 4, 5, 11 and 12) or aged (lanes 6, 7, 13 and 14) *C.*
706 *parvum* oocysts were incubated with 150 μM PMA for 5 min and then exposed to light for 2 min
707 (lanes 3, 5, 7, 10, 12, and 14). Genomic DNA was purified as described in the Materials and
708 Methods. *SSU rRNA* PCR amplicons from live, dead or aged oocysts with an oocysts equivalent

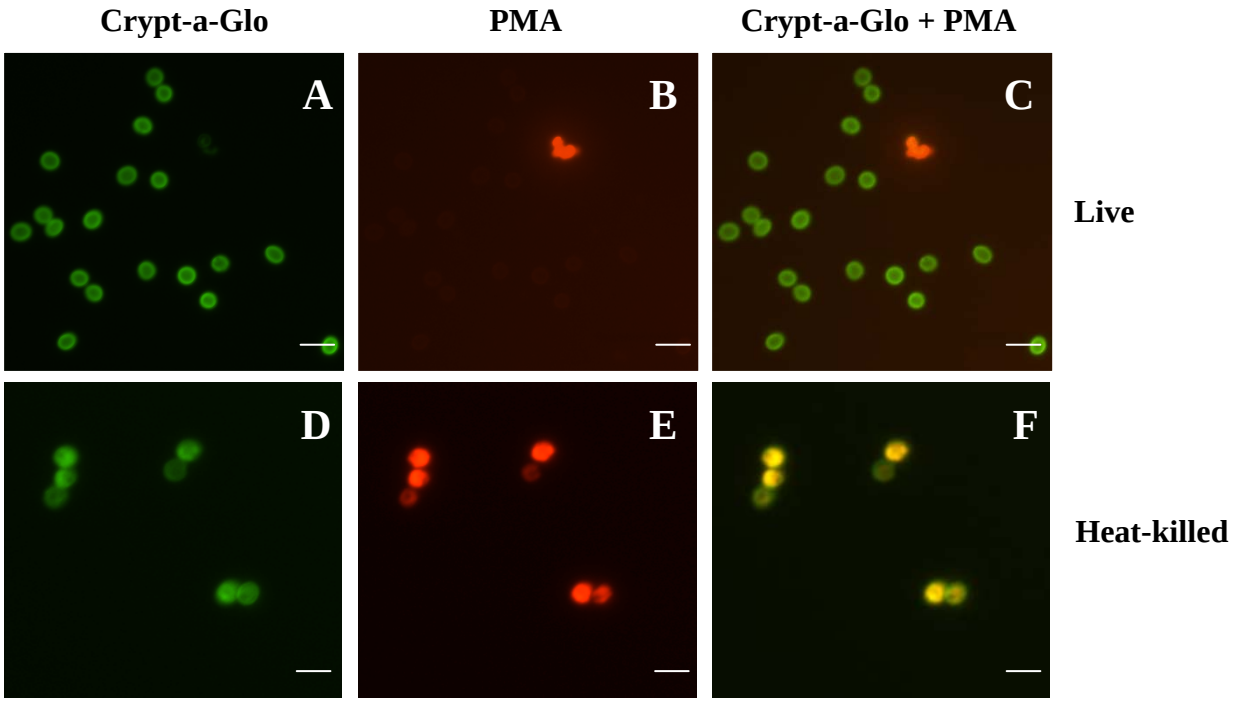
709 of (A) 100 or (B) 10 were visualized using an Agilent Bioanalyzer 2100. M: Marker, - : No

710 PMA, +: PMA added, Δ: 70 ºC treated.

711 Figure 1

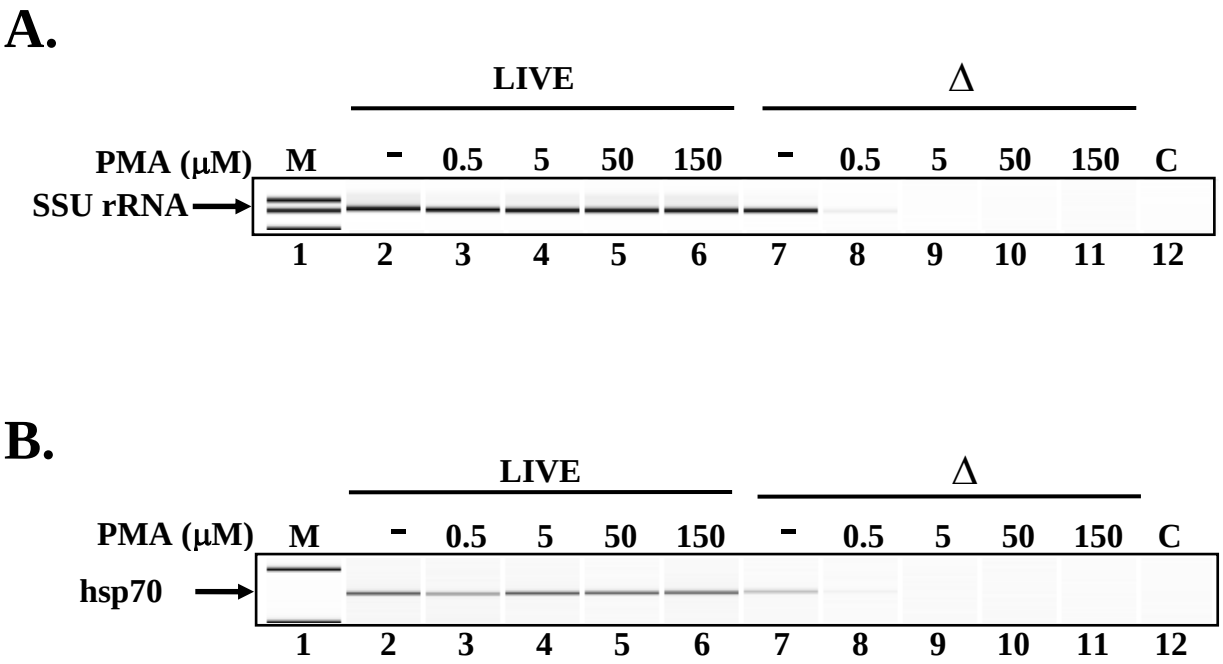
712

713

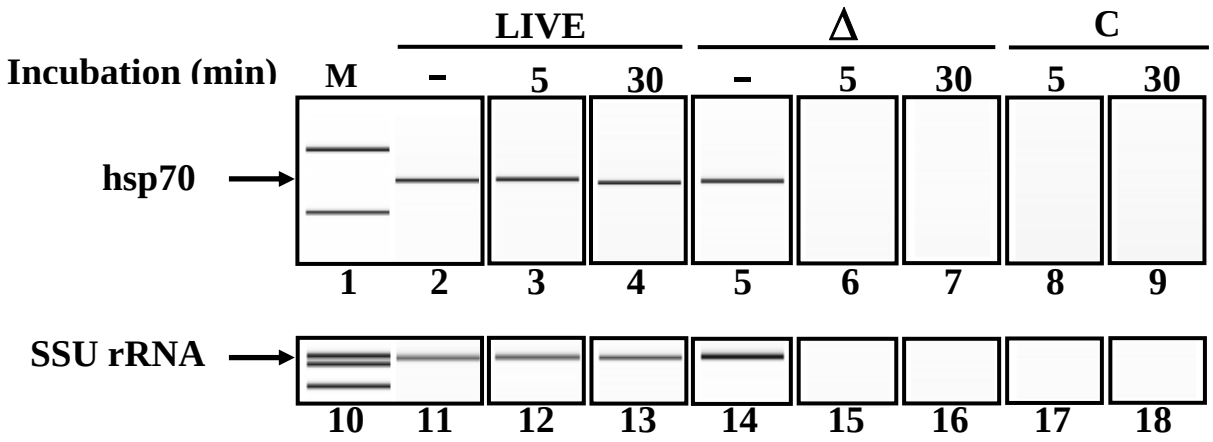


714

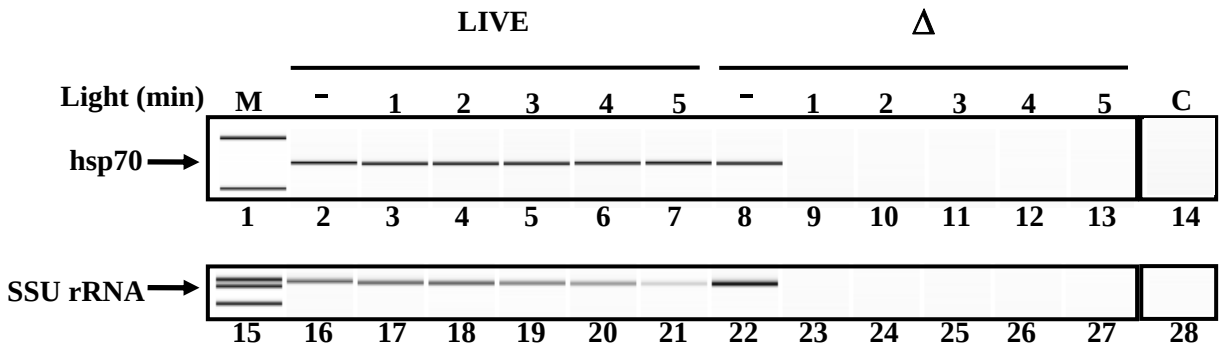
715 Figure 2


A.
LIVE
Δ
PMA (μM)
M
-
0.5
5
50
150
-
0.5
5
50
150
C
SSU rRNA
1 2 3 4 5 6 7 8 9 10 11 12
B.
LIVE
Δ
PMA (μM)
M
-
0.5
5
50
150
-
0.5
5
50
150
C
hsp70
1 2 3 4 5 6 7 8 9 10 11 12

716 Figure 3


LIVE
Δ
C
Incubation (min)
M
–
5
30
–
5
30
5
30
hsp70
1
2
3
4
5
6
7
8
9
SSU rRNA
10
11
12
13
14
15
16
17
18

717 Figure 4


LIVE
Δ
Light (min)
M
-
1
2
3
4
5
-
1
2
3
4
5
C
hsp70
1
2
3
4
5
6
7
8
9
10
11
12
13
14
SSU rRNA
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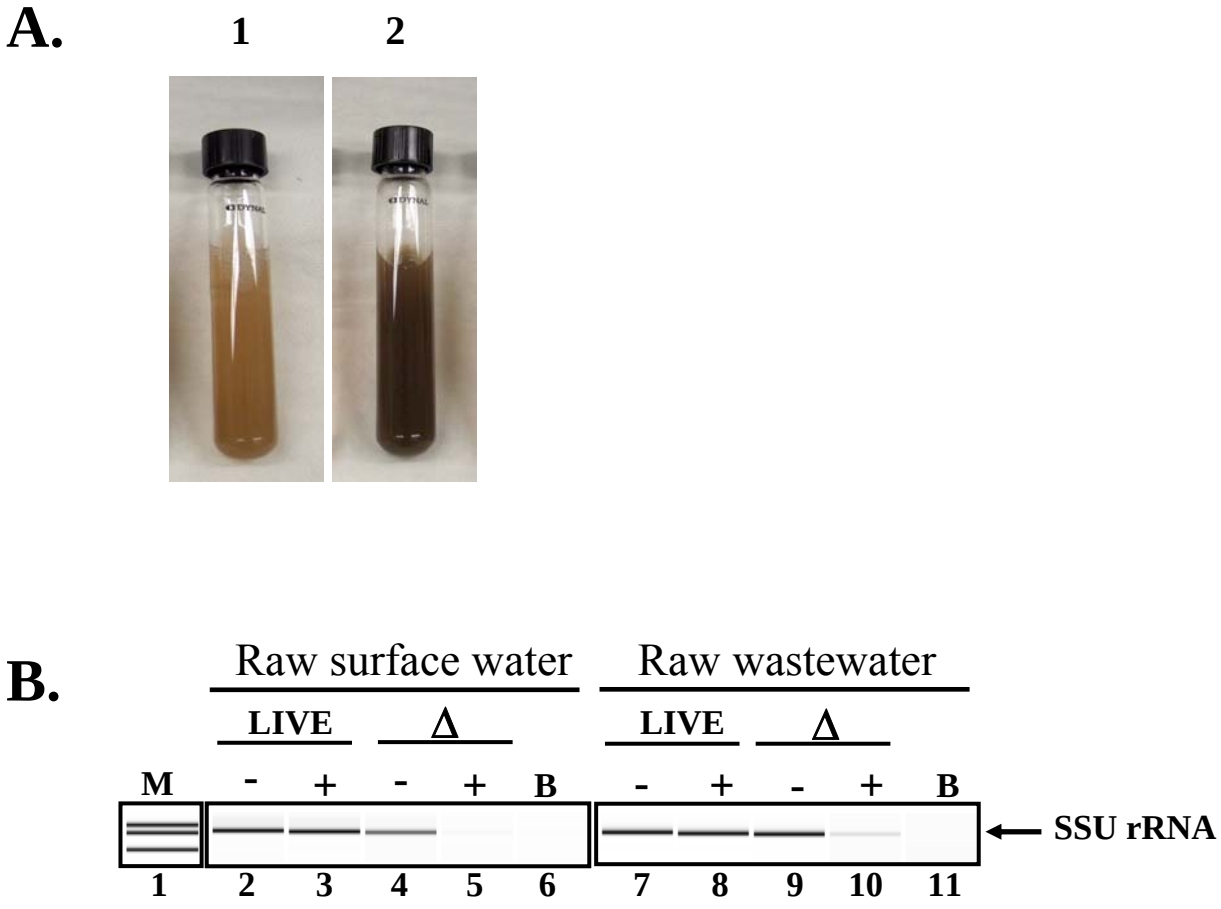
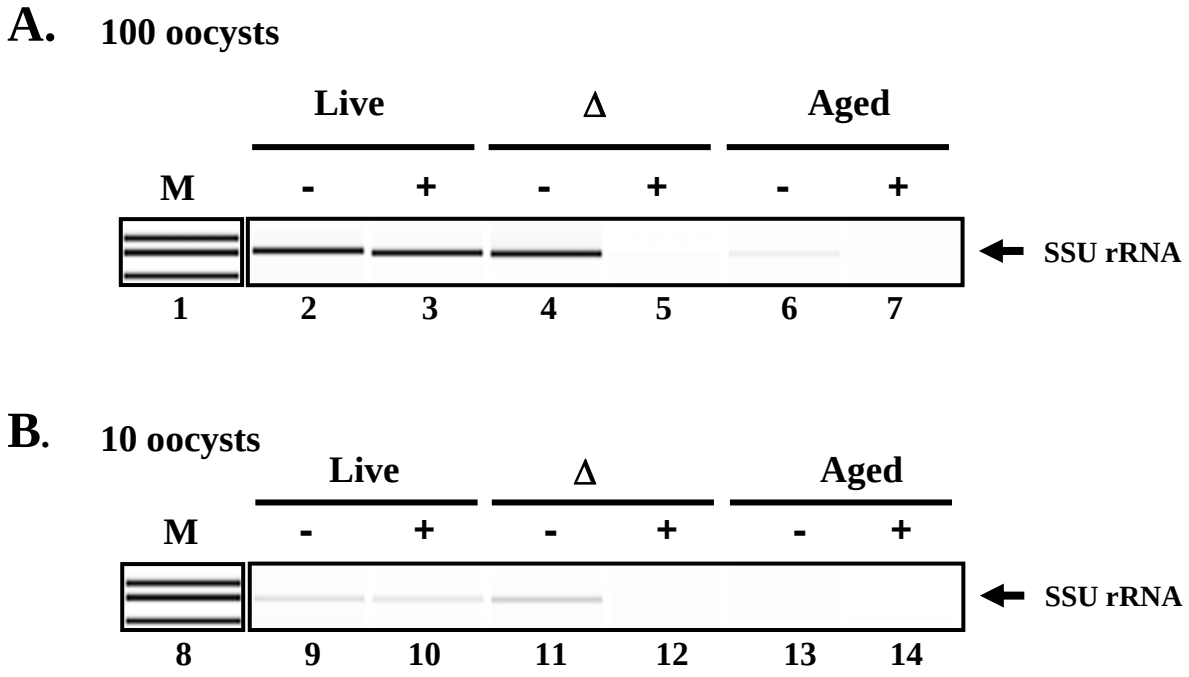
28

718 Figure 5


A.
1
2
B.
Raw surface water
Raw wastewater
LIVE
Δ
LIVE
Δ
M
-
+
-
+
B
-
+
-
+
B
SSU rRNA
1
2
3
4
5
6
7
8
9
10
11

719 Figure 6

720



721 TABLE 1: Molecular genotyping of *C. muris* (Cm) and *C. parvum* (Cp) in a mixture of

722 live and heat-killed oocysts using CryptoPMA-PCR*

723

Oocyst		Ratio	Experiment			Live oocysts detected	Dead oocysts detected
Live	**Heat-killed**		**1**	**2**	**3**		
C. parvum	*C. muris*	0:100	-	-	-	**0/3**	**0/3**
C. parvum	*C. muris*	1:99	-	-	-	**0/3**	**0/3**
C. parvum	*C. muris*	10:90	Cp	-	Cp	**2/3**	**0/3**
C. parvum	*C. muris*	50:50	Cp	Cp	Cp	**3/3**	**0/3**
C. parvum	*C. muris*	100:0	Cp	Cp	Cp	**3/3**	**0/3**
C. muris	*C. parvum*	0:100	-	-	-	**0/3**	**0/3**
C. muris	*C. parvum*	1:99	Cm	-	-	**1/3**	**0/3**
C. muris	*C. parvum*	10:90	Cm	Cm	Cm	**3/3**	**0/3**
C. muris	*C. parvum*	50:50	Cm	Cm	Cm	**3/3**	**0/3**
C. muris	*C. parvum*	100:0	Cm	Cm	Cm	**3/3**	**0/3**

724 **SSU rRNA* and *hsp70* genes were amplified and sequenced.