

Density of environmental *Acanthamoeba* and their responses to superheating disinfection

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Abstract Exposure to viable *Acanthamoeba* may cause fatal encephalitis and blinding keratitis in humans. Quantification of environmental *Acanthamoeba* by a reliable analytical assay is essential to assess the risk of human exposure and efficacy of control measures (e.g., superheating). Two DNA binding dyes (ethidium monoazide (EMA) and propidium monoazide) coupled with real-time quantitative PCR (qPCR) were tested for the ability in selectively quantifying viable *Acanthamoeba castellanii*. This newly developed qPCR assay was applied to determine the density of environmental *Acanthamoeba* and disinfection efficacy of superheating. Results showed qPCR with 2.3 µg/mL EMA performed optimal with a great linearity ($R^2=0.98$) and a wide range of detection ($5-1.5 \times 10^5$ cells). EMA-qPCR analyses on water samples collected from cooling towers, eyewash stations, irrigated farmlands, and various wastewater treatment stages further showed viable *Acanthamoeba* density from nondetectable level to 6.3×10^5 cells/L. Superheating *A. castellanii* at 75–95 °C for 20 min revealed significant reductions in both EMA-qPCR and qPCR detectable *Acanthamoeba* target sequences with an adverse

association between heating temperature and qPCR-determined DNA quantity ($r=-0.76$ to -0.93 , $p<0.0001$). Moreover, *A. castellanii* trophozoites were more sensitive to superheat stress than the cells being encysted for 6 and 13 d ($p<0.05$). This is the first study to quantify environmental *Acanthamoeba* and characterize their responses to superheating by EMA-qPCR. The quantitative data provided in this study facilitate to understand better the relative risk for human exposed to viable *Acanthamoeba* and the efficacy of superheating against *Acanthamoeba*.

Introduction

Acanthamoeba species are recognized as important protozoa mainly because of their health implications in humans and ecological roles in prokaryotes. It is well known that viable *Acanthamoeba* may cause severe human infections leading to blinding keratitis and lethal granulomatous amoebic encephalitis (Khan 2006). In addition, *Acanthamoeba* behaves as a natural reservoir for at least 46 bacteria (Greub and Raoult 2004) and can further enhance the survival, virulence, amplification, and transmission of parasitological bacteria, such as pathogenic *Legionella pneumophila* (Barker et al. 1992, 1995; Berk et al. 1998; Cirillo et al. 1999). This free-living protozoan has a typical two-stage life cycle, i.e., vegetative trophozoites and double-walled cysts. The trophozoites may actively feed on bacteria and protists as food sources while cysts can survive in hostile conditions (Khan 2006). *Acanthamoeba* has been detected in various aquatic environments, including household water (Stockman et al. 2011), cooling tower water (Chang et al. 2010; Pagnier et al. 2009), sewage (Magnet et al. 2012), rice paddy water (Sheng et al. 2009), thermal spring water (Kao et al. 2013), seawater (Lorenzo-Morales et al. 2005; Mahmoudi et al. 2012), and tap water from eyewash stations (Paszko-Kolva et al. 1998) and hospitals (Bagheri et al. 2010). Superheating *Acanthamoeba*-contaminated water

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is considered as one of disinfection treatments to preventing people from waterborne infections in health care settings (Coulon et al. 2010).

Because *Acanthamoeba* impacts human health by its intrinsic pathogenicity and the role as a place of replication and virulence enhancement of waterborne pathogens (Khan 2006; Pagnier et al. 2009), accurate quantification of environmental *Acanthamoeba* is essential to better estimate the risk for human exposed to *Acanthamoeba* and the efficacy of control measures implemented such as superheating. Nevertheless, the conventional assay on *Acanthamoeba* quantification requires serial dilution of samples and culturing of amoeba with seeded bacteria, followed by most-probable-number (MPN) calculations (Behets et al. 2007; Rodriguez-Zaragoza 1994). Such culture procedures are complex, time consuming (weeks), and incapable of detecting amoeba that does not excyst (Riviere et al. 2006). Indeed, the information on the density of environmental *Acanthamoeba* quantified by culture assay is very limited (Behets et al. 2007; Rodriguez-Zaragoza 1994) although the quantity of *Acanthamoeba* articles have been substantially increased in the past 50 years (Khan 2006).

To overcome the limitations of culture assay, real-time quantitative polymerase chain reaction (qPCR) technique has been recently applied to *Acanthamoeba* and validated with environmental samples (Chang et al. 2010; Chang and Wu 2010). The qPCR is a rapid and sensitive tool; however, it detects the DNA regardless of amoebic viability and thereby could overestimate the infectious risk of *Acanthamoeba* in certain scenario (for example, after superheating disinfection). The overestimation concern could be resolved by treating samples with DNA binding dyes such as ethidium monoazide (EMA) and propidium monoazide (PMA) prior to qPCR. Under an optimal setting, the DNA binding dye is expected to exclusively enter cytoplasmic membrane-compromised (nonviable) cells, form covalent bonds with DNA under photoactivation and inhibit DNA amplification in the following qPCR; thus, only the DNA of membrane-intact (viable) cells is amplifiable and quantified by qPCR (Chen and Chang 2010). This viable qPCR technique has been applied to food (Andorra et al. 2010; Lee and Levin 2009), water (Brescia et al. 2009; Chen and Chang 2010), biofilm (Chen and Chang 2010; Pan and Breidt 2007), and air samples (Chang and Chou 2011a, b; Chang and Hung 2012a, b).

It should be noted that the viable qPCR assay needs to be optimized to efficiently suppress qPCR signals from nonviable cells and exclude the dye from live ones (Fittipaldi et al. 2011). A failure in optimization may bias cell counts. Indeed, EMA has been revealed to enter viable *Helicobacter pylori* (Nam et al. 2011), *Enterobacter sakazakii* (Cawthorn and Witthuhn 2008), and *Listeria monocytogenes* (Pan and Breidt 2007), resulting in an underestimation of viable counts. Conversely, PMA fails to completely prevent

DNA amplification from nonviable *L. pneumophila* (Chen and Chang 2010) and mixed bacterial flora of fish fillets (Lee and Levin 2009), thereby overestimating viable loads. Literature has shown the optimal condition of viable qPCR, in terms of the type and concentration of DNA dye, may differ among microorganisms (Fittipaldi et al. 2011). This highlights the importance of a comparative evaluation on both DNA dyes when developing a viable qPCR assay, which has not been simultaneously tested for *Acanthamoeba*.

The present study was undertaken to determine an optimal viable assay for *Acanthamoeba* by comparing the performance between PMA- and EMA-qPCR on *Acanthamoeba castellanii* trophozoites and cysts. With the viable qPCR and qPCR methods, the densities of viable and total *Acanthamoeba* were respectively quantified from the water of cooling towers, eye-wash stations, irrigated farmlands and different treatment stages of a wastewater treatment plant (WWTP). The viability ratio, defined as the ratio of viable to total *Acanthamoeba* cell counts, was also determined for the first time. Additionally, considering *Acanthamoeba* may exist in the water supply systems of hospital (Bagheri et al. 2010; Fields et al. 1989) where superheating is commonly adopted as one of control measures, the disinfection efficacy of superheating was also evaluated against *A. castellanii* trophozoites and cells being encysted for 6 and 13 days. *A. castellanii* was tested because it is one of the most widespread and commonly isolated species (Khan 2006).

Materials and methods

Acanthamoeba strain and culture condition

A. castellanii (ATCC 30234) trophozoites were grown in proteose–yeast–glucose medium (PYG) 712 at 25 °C for 3 days (Chang et al. 2010). For encystment, *A. castellanii* trophozoites (40 mL) were centrifuged at 200×g for 8 min, re-suspended in 10 mL of encystment medium (0.1 M KCl, 8 mM MgSO₄, 0.4 mM CaCl₂, 1 mM NaHCO₃, and 0.02 M Tris), washed twice with 10 mL encystment medium by centrifugation (200×g, 8 min), and incubated at 25 °C (Riviere et al. 2006) for 6 and 13 days. *A. castellanii* cysts, characterized as round shape with two-wall layer (Page 1988), were counted on the 6th and 13th days with a hemocytometer (Marienfeld, Lauda-Konigshofen, Germany) under a phase contrast microscope (Leica DM2500, Wetzlar, Germany) at ×400. The cyst percentage was determined 53.6±3.5 % and 91.7±1.8 % in 6- and 13-day encystment cultures, respectively ($n=3$, data not shown); thereby, they were designated as cells at transient stage (from trophozoites to cysts) and cysts, respectively.

A. castellanii trophozoites were centrifuged at 200×g for 8 min, whereas cysts and cells at transient stage were

recovered by centrifugation at $2,000\times g$ for 5 min (Riviere et al. 2006). Amoebic pellets were added with 10 mL sterile Page's amoeba saline (PAS; 120 mg NaCl, 4 mg $MgSO_4 \cdot 7H_2O$, 4 mg $CaCl_2$, 142 mg Na_2HPO_4 , and 136 mg KH_2PO_4 in 1 L ultrapure water), centrifuged and re-suspended in 10 mL PAS, and counted by a hemocytometer (Marienfeld) under a phase contrast microscope (Leica) at $\times 400$. Serial dilution was performed to adjust amoebic concentrations for the following tests.

Heat challenge and culture on NNA plates

Exposure to high temperature may inactivate *Acanthamoeba* (Coulon et al. 2010). Thus, heat-treated *A. castellanii* were prepared along with unheated cells and used to evaluate the ability of EMA and PMA in discriminating and quantifying viable *Acanthamoeba* in qPCR. In practice, *A. castellanii* trophozoites (20 mL) in PAS were heated at 75 °C for 20 min in a water bath (FIRSTEK, Taipei, Taiwan), whereas cysts were heated at 95 °C owing to their resistant characteristic.

To determine amoebic viability, heated and unheated cells (0.05 mL) were inoculated triplicate on the center of non-nutrient agar (NNA) plates pre-seeded with heat-killed (70 °C, 2 h) *Escherichia coli*, followed by an incubation at 25 °C for 7 and 14 days for trophozoite and cyst cultures, respectively. The NNA plates were observed daily under an inverted microscope (Eclipse TE2000-U, Nikon, Tokyo, Japan) at $\times 100$ to determine the presence of trophozoites. The diameter of clearing zone on NNA, as an indicator of amoebic feeding on *E. coli*, was also measured.

EMA and PMA treatment

EMA (Sigma Chemical Co., St. Louis, MO, USA) was diluted in sterile water. PMA (Biotium, Inc., Hayward, CA, USA) was dissolved in 20 % dimethylsulfoxide (Sigma). Heated and unheated *A. castellanii* trophozoites in PAS (0.5 mL) were reacted with 50 μ L of EMA or PMA at final concentrations of 2.3, 23, and 76.7 μ g/mL, respectively, in the dark for 5 min at room temperature (~ 26 °C), followed by placement on chipped ice and exposure to a halogen light (500 W; OSRAM, Munchen, Germany) at a 15-cm distance for 5, 10, and 20 min. The ice was replenished every 5 min. Heated and unheated cysts (0.5 mL) were also reacted with 50 μ L of EMA or PMA prior to 20-min light exposure (500 W) on ice. Heated and unheated *A. castellanii* without EMA or PMA treatment were prepared as controls. The experiments were repeated at least three times. Samples were then processed for DNA extraction and qPCR as described below in the section of DNA extraction and qPCR.

Linearity and limits of detection

A. castellanii trophozoites were serially diluted with PAS to obtain amoebic concentrations between 3 and 3×10^6 cells/mL. Trophozoites (1 mL) of various concentrations were heated at 75 °C for 20 min. Heated and unheated cells (0.5 mL) were respectively treated with 2.3 μ g/mL of EMA following the procedures described above. Cells without EMA treatment were prepared as controls. The experiments were repeated four times to determine the range of linearity, R^2 (coefficient of determination) and limits of detection for EMA-qPCR assay.

Effects of superheat challenge

To determine the superheating efficacy against *Acanthamoeba*, *A. castellanii* trophozoites, cysts, and cells at transient stage were adjusted to 3×10^5 cells/mL in PAS, followed by 20-min heat challenge in a water bath (FIRSTEK) set at 75, 80, 85, 90, and 95 °C, respectively. Unheated cell suspension was also prepared as controls. Heated and unheated *A. castellanii* (0.5 mL) were processed with or without EMA staining (2.3 μ g/mL), followed by DNA extraction and qPCR as described below. The experiments were repeated four times.

Field sampling and pretreatment processing

Thirty water samples were collected from eyewash stations of research laboratories (1 L, $n=6$), cooling towers of a university (1 L, $n=6$), an influent site (0.5 L) and exits of secondary clarifier (0.1 L) and chlorination basin (1 L) of a WWTP ($n=6$), irrigated fields ($n=6$) and surrounding ditches ($n=6$) of rice farmlands (0.1–0.2 L). Samples obtained from eyewash station, cooling tower and chlorination basin were added with 1 mL of 10 % (v/v) $Na_2S_2O_3$ to neutralize the residual chlorine. All samples were transported at 4 °C and processed immediately in the laboratory by centrifugation ($9,000\times g$, 10 min), reaction with and without EMA of 2.3 μ g/mL, DNA extraction and qPCR.

DNA extraction and qPCR

DNA of *Acanthamoeba* was extracted by FastDNA[®] Spin kit for soil (MP biomedical, Irvine, CA, USA). In brief, amoeba suspended in PAS was centrifuged at $14,000\times g$ for 10 min, re-suspended in 978 μ L sodium phosphate buffer, processed with bead beating, and DNA extraction following the manufacturer's instructions, except for extending the bead beating time to 1 min and increasing the volume of DNA elution solution to 100 μ L (Chang et al. 2010; Chang and Wu 2010).

To relieve PCR inhibition, DNA extracted from environmental samples were diluted with TE buffer (i.e., a buffer containing Tris and EDTA) prior to qPCR. The qPCR was

undertaken in a LightCycler 480 (Roche Diagnostics, Mannheim, Germany). A 180-bp DNA segment on *Acanthamoeba* 18S rDNA was quantified by the primers and probe designed by Qvarnstrom et al. (2006). The reaction mix (25 μ L) contained 0.24 μ M forward primer (5'-CCCAGATCGTTTACCGTGAA-3'), 0.24 μ M reverse primer (5'-TAAATATTAATGCCCCCAACTATCC-3'), 0.24 μ M probe (5'-FAM-CTGCCACCGAATACATTAGCATGG-BHQ1-3'), 10 μ L LightCycler FastStart DNA Master Hybridization Probes mix (Roche Diagnostics), 10 μ L extracted or standard DNA and PCR grade sterile water (Roche Diagnostics). The thermal program was set as 95 $^{\circ}$ C for 10 min, followed by 50 cycles at 95 $^{\circ}$ C for 15 s and 52 $^{\circ}$ C for 1 min, and a final cooling at 40 $^{\circ}$ C for 15 s. Each run of qPCR consisted of test samples, serially diluted DNA standards and no template control. DNA standards that contained 2,728-bp plasmid yT&A vector carrying a 180-bp PCR product from a fragment of *A. castellanii* (ATCC 30234) DNA amplified using the primers and qPCR as described above were synthesized by Mission Biotech Co., Ltd (Taipei, Taiwan).

A qPCR calibration curve of DNA standards (Ct value vs. log fg/ μ L) was constructed and used to determine the DNA concentration in test samples. The resulting DNA concentrations (log fg/ μ L) were anti-log transformed, multiplied by elution volume (100 μ L), ratio of *A. castellanii* DNA bp to total bp of DNA standard (i.e., 180/2,908) and DNA dilution factor (environmental samples only), and further adjusted for sample volume to determine the log DNA quantity of *Acanthamoeba* in test samples (log fg/sample).

Acanthamoeba counts in environmental samples

Cell counts of viable and total *Acanthamoeba* in environmental samples were determined based on two calibration curves constructed using serially diluted and unheated *A. castellanii* cells pre-treated with and without EMA of 2.3 μ g/mL, respectively, i.e., Y (log fg/sample) = 1.12 X (log cells/sample) - 1.70 for viable cells; Y = 1.17 X - 2.01 for total cells (Fig. 3a). In practice, the greatest log DNA quantity among undiluted and diluted DNA of each environmental sample was input as the Y value in the abovementioned equations. Resulting log cell count (X) was anti-log transformed and adjusted for sample volume to obtain the respective cell concentration (cells per liter). A ratio of viable to total cell counts (i.e., viability ratio) was also determined.

Data analysis

The nonparametric Wilcoxon rank-sum test was performed to examine the difference in DNA quantity between EMA- and PMA-treated samples and between the samples with and without EMA. The Kruskal–Wallis test with the post hoc Scheffé's test were applied to examine the differences in

DNA quantity among (1) samples with EMA/PMA at various dye concentrations and photoactivation durations, (2) heated and EMA-treated trophozoites over 6 orders of magnitude of cell counts, and (3) EMA-treated cells at 25–95 $^{\circ}$ C exposure. The reduction levels in log DNA quantity of 75–95 $^{\circ}$ C heated cells relative to unheated ones were also calculated and statistically examined by Scheffé's test for the difference among trophozoites, cysts, and cells at transient stage. Moreover, Spearman rank correlation was used to explore the relationship between heating temperature and qPCR-determined DNA quantity. The difference between viable and total counts of *Acanthamoeba* in environmental samples was examined by Wilcoxon signed rank test. All analyses were conducted using SAS software version 9.1 (SAS Institute Inc., NC, USA). Statistical significance was considered as $p < 0.05$.

Results and discussion

Amoebic viability on NNA plates

Microscopic observation revealed abundant trophozoites in unheated amoebic suspensions, with an increase in the mean diameter of clearing zone on NNA plates from 2.1 cm (1st day of incubation) to 6.6 cm (7th day) for trophozoite suspensions and to 8.5 cm (14th day) for cyst cultures (data not shown). Full of trophozoites and an increase in clearing zone confirmed active feeding of *Acanthamoeba* on *E. coli* prey and the dominance of viable *A. castellanii* in unheated cultures. By contrast, trophozoites were not found in any of samples preheated at ≥ 75 $^{\circ}$ C and an expansion of clearing zone was absent, demonstrating a detrimental effect of superheating on *A. castellanii*. Heated and unheated *A. castellanii* were thus used to test the EMA/PMA discriminability in qPCR.

EMA/PMA-qPCR on trophozoites

Within tested EMA concentrations (2.3–76.7 μ g/mL) and photoactivation times (5–20 min), Fig. 1a shows the qPCR-determined DNA quantities averaged 4.2–4.7 log fg in unheated and EMA-treated cells (grey bars), similar to 4.5 log fg of unheated cells but no EMA treatment. In contrast, the mean DNA quantities in heated and EMA-treated trophozoites varied between -0.9 and -0.01 log fg, significantly lower than that of heated cells without EMA by 3.8–4.7 log fg ($p < 0.05$) (black bars, Fig. 1a). Similar results were also revealed in PMA testing (Fig. 1b), i.e., the DNA quantities of unheated cells were statistically comparable regardless of PMA treatment (4.3–4.8 log fg, $p > 0.05$), whereas the mean DNA quantities in heated and PMA-treated cells (-0.8 to 0.07 log fg) were significantly lower than that without PMA (3.8 log fg; $p < 0.05$).

Comparable DNA quantities among unheated trophozoites (Fig. 1) illustrate neither EMA nor PMA interfered with the quantification of viable trophozoites by qPCR. By contrast, significant decreases in DNA quantities of heated trophozoites pre-reacted with EMA/PMA show the ability of both DNA dyes in minimizing qPCR signals from nonviable trophozoites. Further statistical analyses on the data of heated and unheated trophozoites, respectively, showed comparable DNA quantities among EMA- or PMA-treated cells regardless of dye concentration and photoactivation time (all $p > 0.05$), indicating that an increase of EMA/PMA concentration from 2.3 to 76.7 $\mu\text{g/mL}$ and photoactivation time from 5 to 20 min did not significantly affect the discriminability of both dyes in viable *A. castellanii*.

EMA/PMA-qPCR on cysts

Since photoactivation time of 5–20 min showed comparable DNA quantities in qPCR (Fig. 1), *A. castellanii* cysts were evaluated with DNA dyes under a 20-min light exposure. Figure 2 shows comparable DNA quantities between unheated cysts with and without EMA/PMA of 2.3 $\mu\text{g/mL}$ ($p > 0.05$, grey bars); however, unheated cysts treated with 23 $\mu\text{g/mL}$ of PMA or EMA had mean DNA quantities significantly lower than those without EMA/PMA by approx. 1 log fg ($p < 0.05$). This finding suggests that EMA and PMA at 23 $\mu\text{g/mL}$ entered a portion of viable cysts probably via the holes known as ostioles (Chavez-Munguia et al. 2005), formed covalent bonds with DNA under photoactivation and blocked qPCR amplification. Similar observation has also been documented in bacterial endospores by Mohapatra and La Duc (2012) who revealed the concentration of live *Bacillus pumilus* spores pre-treated with 44 μM (22.5 $\mu\text{g/mL}$) of PMA was decreased by 1.18 log spores/mL as compared with the spores without PMA treatment.

As for 95 °C heated cysts (black bars, Fig. 2), they were not detected by qPCR following reactions with EMA or PMA of 2.3 and 23 $\mu\text{g/mL}$, whereas heated cysts without EMA/PMA averaged 0.9 log fg, illustrating that 2.3 $\mu\text{g/mL}$ EMA or PMA was sufficient to inhibit DNA amplification from nonviable cysts in qPCR.

To examine the overall performance between EMA-qPCR and PMA-qPCR, statistical testing was conducted on the data of Figs. 1 and 2. DNA quantities between EMA- and PMA-treated samples were found comparable for trophozoites and cysts (all $p > 0.05$), highlighting similar efficiencies of EMA-qPCR and PMA-qPCR in *Acanthamoeba* quantification. Equivalent performance between EMA- and PMA-qPCR has also been reported in wine yeast (Andorra et al. 2010).

In summary, Figs. 1 and 2 clearly indicate that treatment of *A. castellanii* with EMA or PMA at 2.3 $\mu\text{g/mL}$ exhibited no interference in DNA amplification of live cells but significantly reduced qPCR signal by 4 logs or presented non-detectable

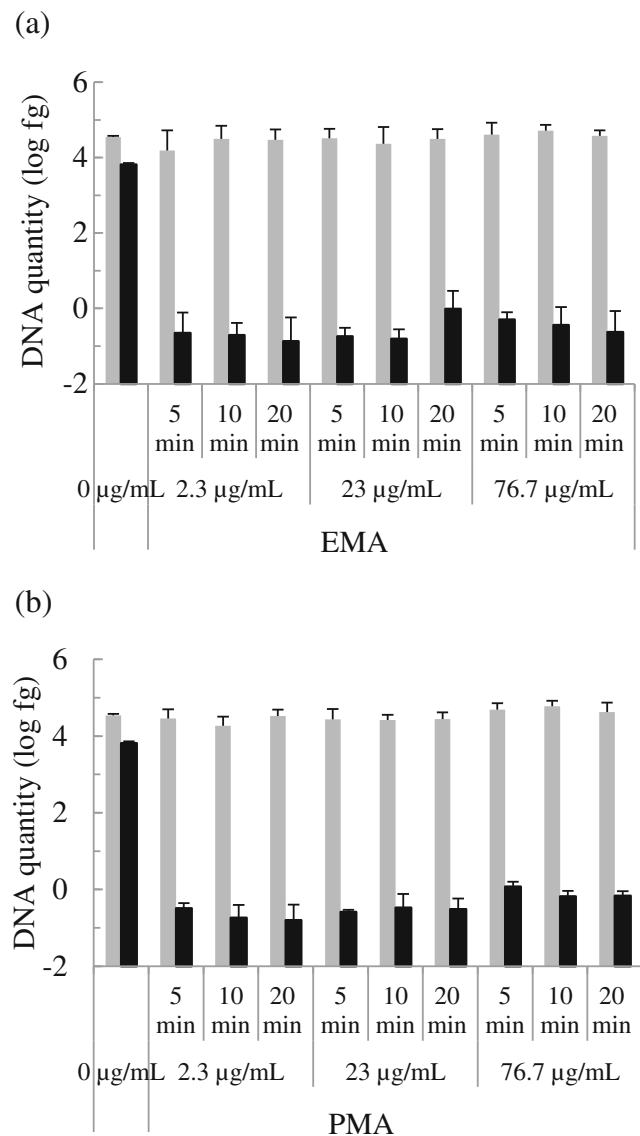


Fig. 1 Effects of concentration of **a** EMA and **b** PMA and exposure time (5–20 min) to a 500-W halogen light on DNA quantity determined by qPCR from unheated (grey) and 75 °C heated (black) trophozoites of *A. castellanii* ($n=4$)

level for nonviable cells. In contrast, DNA quantities of viable cysts were underestimated when treating cells with 23 $\mu\text{g/mL}$ EMA or PMA. Moreover, the light exposure time of 5–20 min did not significantly affect *Acanthamoeba* quantification. Considering our findings and a longer period of time exposure (10–30 min) to a 500-W light was often performed at low EMA concentrations (≤ 2.5 $\mu\text{g/mL}$) (Lee and Levin 2008; Luo et al. 2008), EMA of 2.3 $\mu\text{g/mL}$ with 20-min light exposure was adopted as optimal for *Acanthamoeba*.

Limits of detection and linearity of EMA-qPCR

The optimal EMA-qPCR was applied to serially diluted *A. castellanii* to determine the limits of detection and linearity

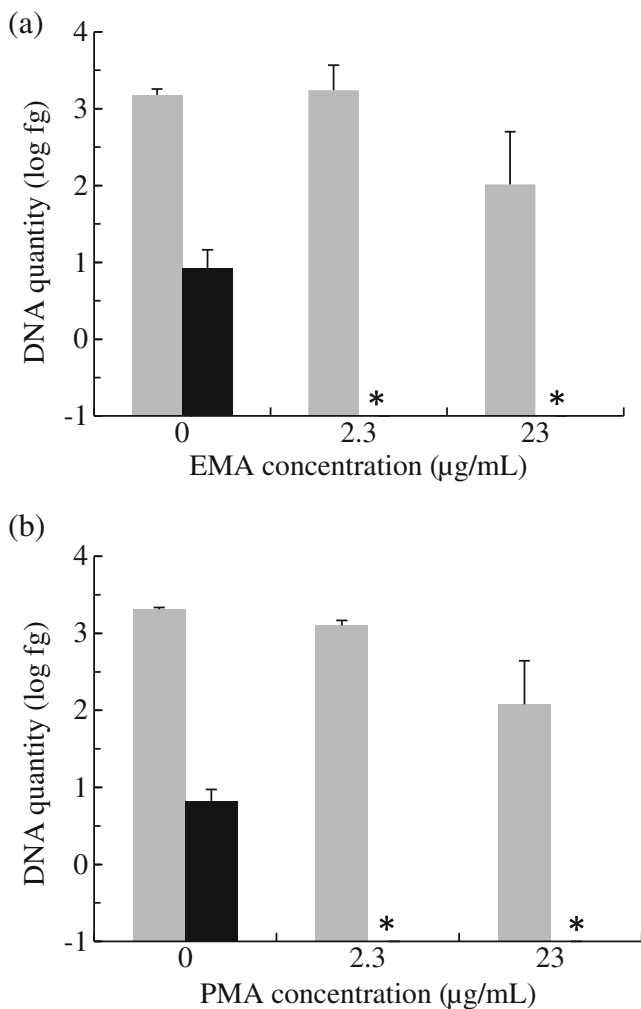


Fig. 2 Effects of concentration of **a** EMA and **b** PMA on DNA quantity determined by qPCR from unheated (■) and 95 °C heated (■) cysts of *A. castellanii* ($n=3$). Asterisk denotes nondetected

with cell counts. For unheated cells treated with EMA (solid diamonds, Fig. 3a), the DNA quantity was linearly increased with cell counts over 7 orders of magnitude (Y (log fg/sample) = $1.12X$ (log cells/sample) - 1.70, $R^2=0.98$). A linearity was also observed for unheated cells without EMA treatment (hollow diamonds, Fig. 3a, $Y=1.17X-2.01$, $R^2=0.95$). Moreover, statistically comparable DNA quantities were observed between EMA-treated and EMA-untreated cells ($p>0.05$), illustrating EMA of 2.3 µg/mL did not interfere with qPCR performance in quantification of live *A. castellanii* over a wide range of cell counts.

For heated and EMA-treated cells (solid diamonds, Fig. 3b), their DNA quantities were significantly lower than those without EMA (hollow diamonds, $p<0.05$). Moreover, heated and EMA-treated *A. castellanii* at a range of $1.5-1.5 \times 10^5$ cell counts presented low and statistically comparable DNA quantities ($p>0.05$) with an average of -0.89 log fg, demonstrating that EMA of 2.3 µg/mL effectively blocked

qPCR signals from nonviable *A. castellanii* of $\leq 1.5 \times 10^5$ cells. However, an increase of DNA quantity up to 3.5 log fg was observed for the samples containing more than 1.5×10^5 heated and EMA-treated cells, indicating an incomplete inhibition of DNA amplification by EMA; thereby, a portion of nonviable cells were detected by qPCR. Considering an optimal EMA-qPCR should effectively prevent DNA amplification from nonviable cells and accurately quantify live ones, the upper limit of detection (ULD) was determined as 1.5×10^5 cells according to Fig. 3a, b. This ULD value is 30 times that the highest count of total *Acanthamoeba* (viable plus nonviable cells) available in the literature (5×10^3 cells/sample, Chang et al. 2010).

With regard to lower limit of detection (LLD), the mean DNA quantity (-0.89 log fg) determined by EMA-qPCR for nonviable *A. castellanii* of $\leq 1.5 \times 10^5$ cell counts was input as Y in the calibration curve of $Y=1.12X-1.70$ (Fig. 3a), resulting in a LLD of 5 cells/sample after anti-log calculation. This LLD value is very close to the LLD of qPCR developed for total *Acanthamoeba* (three cells, Chang et al. 2010) and lower than that of PMA-PCR for another protozoan, *Cryptosporidium* (ten oocysts, Brescia et al. 2009).

Acanthamoeba density and viability ratio in aquatic environments

High R^2 (0.95–0.98) of calibration curves shown in Fig. 3a strongly support both curves, constructed respectively by EMA-qPCR and qPCR, can be used to convert qPCR-determined DNA quantities to viable and total *Acanthamoeba* counts, as being performed in previous studies (Berrada et al. 2006; Chang et al. 2010). By doing such transformation for 20 *Acanthamoeba*-positive environmental samples, Table 1 shows cell concentrations of viable *Acanthamoeba* were consistently lower than those of total cells regardless of sampling site ($p<0.05$) and the viability ratio ranged from 0.11 to 0.94. This finding not only demonstrates EMA-qPCR can be used to rapidly and reliably determine viable *Acanthamoeba* loads in various kinds of aquatic environments but also highlights 11–94 % of *Acanthamoeba* cells were actually viable. To our knowledge, this is the first study to show the density of viable *Acanthamoeba* by EMA-qPCR and to present the data of viability ratio, which is valuable for further research on exposure assessment of *Acanthamoeba* as only viable *Acanthamoeba* may cause severe human infections.

In addition, the quantitative data provided in Table 1 allows, as the first time, to understand better the relative risk for human exposed to viable *Acanthamoeba* of various aquatic environments. For instance, viable *Acanthamoeba* of 0–300 cells/L was found in cooling water (i.e., four were *Acanthamoeba*-negative and the other two were 4.9 and 300 cells/L (Table 1)), which is similar to the level of 0–452 cells/L in cooling water of Belgian electrical power plants by culture/

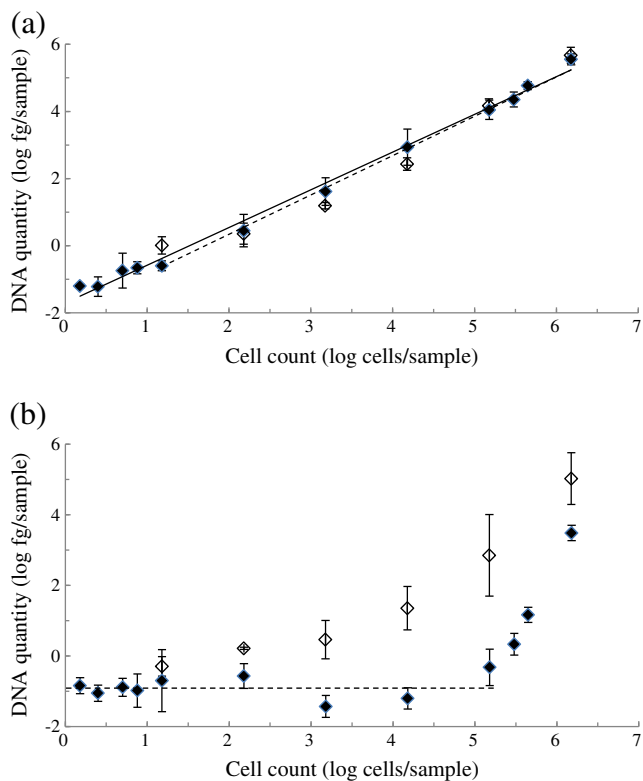


Fig. 3 qPCR-determined DNA quantity of **a** unheated and **b** heated *A. castellanii* trophozoites treated (filled diamonds) or untreated (empty diamonds) with 2.3 $\mu\text{g}/\text{mL}$ EMA ($n=4$). The calibration curves in **(a)** are $Y=1.17X-2.01$ (dotted line, EMA-untreated cells, $R^2=0.95$) and $Y=1.12X-1.70$ (solid line, EMA-treated cells, $R^2=0.98$). A dotted line in **(b)** represents a mean DNA quantity of -0.89 log fg for heated and EMA-treated cells over $1.5-1.5 \times 10^5$ cells/sample

MPN methods (Behets et al. 2007). Compared with cooling water, greater concentrations of viable *Acanthamoeba* were revealed in farmland-associated samples (Table 1), for which viable *Acanthamoeba* was even more abundant in the water of irrigated rice paddies (1.7×10^4 – 4.2×10^4 cells/L) than in the running ditch water used for irrigation (3.2×10^2 – 8.5×10^3 cells/L). *A. castellanii* isolated from the water of a rice field has been linked to the GAE infection in a farmer who accidentally fell into a paddy gully (Sheng et al. 2009), highlighting an exposure to *Acanthamoeba* in farmlands may result in severe infections. However, there were no reference literatures about the density of viable *Acanthamoeba* in the water of farmlands. Our data clearly indicate that, as the first time, viable *Acanthamoeba* were abundant in irrigated rice fields. The farmers should be aware of potentially infectious risks by *Acanthamoeba* and use personal protection equipment whenever appropriate to minimize their exposure.

As *Acanthamoeba* has been frequently detected in the water of WWTPs (Magnet et al. 2012), water sampling was also conducted in two occasions at three sites of a WWTP. The level of viable *Acanthamoeba* at the exit of secondary clarifier

Table 1 Cell concentrations of *Acanthamoeba*-positive water samples collected from cooling towers (C), irrigated fields (F) and surrounding ditches (D) of rice farmlands, influent site (I) and exits of secondary clarifier (SC) and chlorination basin (CB) of a wastewater treatment plant

Sampling	Sample	<i>Acanthamoeba</i> concentration (cells/L) ^b		Ratio
		Viable	Total	
Site (n) ^a	ID			Viable/total
Cooling towers (2)	C-1	4.9×10^0	4.5×10^1	0.11
	C-2	3.0×10^2	4.1×10^2	0.73
Fields of rice farmlands (6)	F-1	2.4×10^4	2.7×10^4	0.88
	F-2	2.9×10^4	3.3×10^4	0.89
	F-3	4.2×10^4	6.1×10^4	0.68
	F-4	3.0×10^4	3.6×10^4	0.82
	F-5	1.7×10^4	5.3×10^4	0.31
	F-6	1.9×10^4	2.1×10^4	0.92
Ditches of rice farmlands (6)	D-1	1.2×10^3	2.6×10^3	0.47
	D-2	1.2×10^3	1.5×10^3	0.78
	D-3	1.3×10^3	1.8×10^3	0.74
	D-4	3.7×10^2	4.7×10^2	0.78
	D-5	3.2×10^2	9.4×10^2	0.35
	D-6	8.5×10^3	1.9×10^4	0.46
Wastewater treatment plant (6)	I-1	4.0×10^2	2.2×10^3	0.18
	SC-1	7.1×10^3	2.7×10^4	0.26
	CB-1	2.6×10^2	2.4×10^3	0.11
	I-2	4.5×10^4	5.8×10^4	0.78
	SC-2	6.3×10^5	6.7×10^5	0.94
	CB-2	5.2×10^2	1.2×10^3	0.44

^a Number of samples

^b Total and viable cells were respectively quantified by qPCR and EMA-qPCR

was consistently greater than that at the influent site by 1 log (SC-1 vs. I-1 and SC-2 vs. I-2; Table 1). As the wastewater was treated in the secondary clarifier following microbial degradation in an aeration basin, *Acanthamoeba* probably preyed on bacteria and/or protists abundant in the aeration basin (Li et al. 2007) and underwent binary division, resulting in a significant increase of amoebic population. By contrast, the lowest viable *Acanthamoeba* density (2.6×10^2 and 5.2×10^2 cells/L) and viability ratio (0.11 and 0.44) were observed at the exit of chlorination basin (CB-1 and CB-2) among three WWTP sampling sites. *Acanthamoeba* was also not detected in six samples collected from eyewash stations and four samples of cooling towers. These results could be partly attributable to chlorine disinfection practice in tap water, cooling tower and chlorination basin, as the literature shows 2-h exposure to 2 mg/L of free chlorine resulted in complete degeneration of trophozoites of *Acanthamoeba polyphaga* (Kilvington 1990).

Efficacy of superheating

A. castellanii subjected to 75–95 °C were analyzed by qPCR and EMA-qPCR to assess the heating effects. The qPCR results showed heating trophozoites at 75–80 and 85–95 °C reduced their DNA quantities by 1.2–1.4 and 2.5–2.7 log fg, respectively, relative to unheated ones at 25 °C (grey bars, Fig. 4a) with a correlation coefficient (r) of -0.76 between heating temperature and DNA quantity ($p < 0.0001$). Such adverse relationship was also observed for cells at transient stage (grey bars, Fig. 4b) and cysts (Fig. 4c) ($r = -0.86$ and -0.77 , respectively, both $p < 0.0001$). A decrease of qPCR-determined DNA quantity with increasing heating temperature strongly supports superheating at 75–95 °C damaged amoebic DNA in a magnitude related to heating strength. DNA degradation and hydrolysis have been noted after heating *Clostridium perfringens* at 75–100 °C (Novak et al. 2005) and also likely occur in 75–95 °C heated *A. castellanii*. The more damage occurred in the DNA of *A. castellanii* by increasing temperature, the less amount of intact DNA available to be detected and quantified by qPCR.

When analyzed by EMA-qPCR, the DNA quantities of 75–95 °C heated trophozoites sharply decreased by 5.1–5.4 log fg compared with that at 25 °C (black bars, Fig. 4a, $p < 0.05$) but were statistically comparable among the challenged 75–95 °C (-1.2 to -1.4 log fg, $p > 0.05$). These results suggest superheating at ≥ 75 °C significantly compromised the integrity of cytoplasmic membrane of trophozoites, thereby allowing EMA penetration and effective reduction in qPCR signals. Moreover, relatively low and comparable DNA quantities among 75–95 °C heated trophozoites strongly support the cytoplasmic membrane of nearly all trophozoites was impaired by 75 °C heating. However, this was not occurred in *A. castellanii* encysted for 6 and 13 days (black bars, Fig. 4b, c). Instead, a strong and adverse association between DNA quantity and heating temperature was revealed ($r = -0.91$ and -0.93 , respectively, both $p < 0.0001$), suggesting that more cysts and cells at transient stage encountered destruction in their cell walls and cytoplasmic membranes as increasing heating temperature from 75 to 95 °C. Increasing temperature is believed to increase the permeability of *Acanthamoeba* cyst wall (Heaselgrave et al. 2006), which would facilitate EMA entrance and inhibition in qPCR signals.

Taking unheated cells as the reference, relative reductions in log DNA quantities determined by qPCR and EMA-qPCR were respectively calculated for heated cells. For DNA quantities measured by qPCR, there were comparable reduction levels between heated cysts and cells at transient stage (i.e., 0.7–1.3, 1.8 and 2.2 log fg at 75–85, 90, and 95 °C, respectively; $p > 0.05$). However, their reduction levels were both significantly lower than those of heated trophozoites (i.e., 1.2–2.5, 2.7, and 2.5 log fg; both $p < 0.05$). Interestingly,

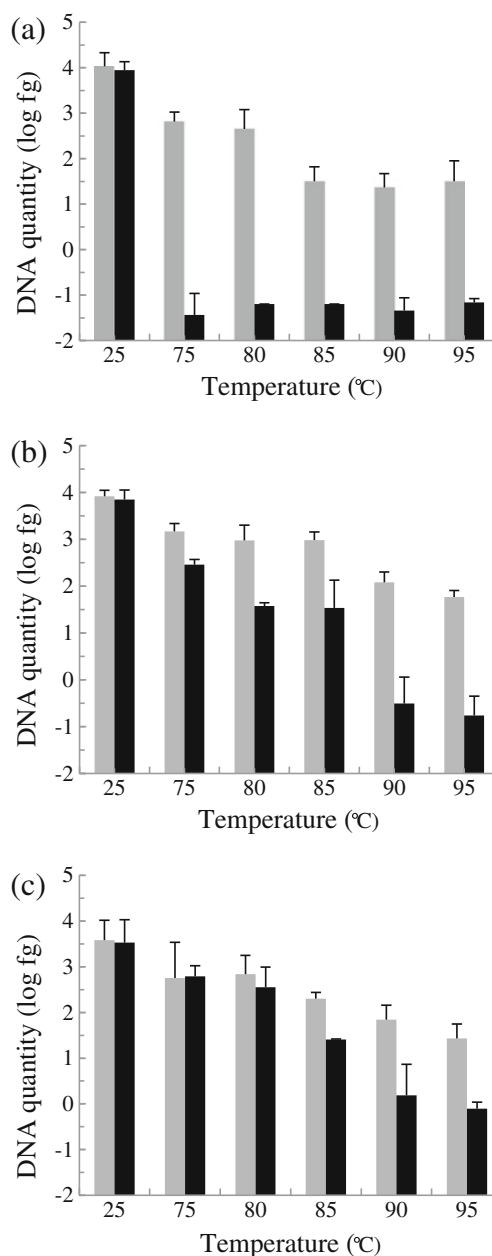


Fig. 4 qPCR-determined DNA quantity for **a** trophozoites of *A. castellanii* and *Acanthamoeba* cultures encysted for **b** 6 and **c** 13 days facing different levels of heat stress (75 to 95 °C, 20 min) and treated with (■) and without (■) EMA ($n = 4$)

similar findings were also observed in the reduction levels derived from the EMA-qPCR (i.e., 0.7–4.6 log fg for cysts and transient cells; 5.1–5.4 log fg for trophozoites; both $p < 0.05$). Consistent lower reduction levels in cysts and cells at transient stage indicate they were more resistant to superheating than trophozoites, which is probably attributable to the development of cyst walls (Turner et al. 2000) and dehydration of protoplast during the encystment transition (Bowers and Korn 1969).

Superheating data further show not all of *A. castellanii* cysts were inactivated at 95 °C, evident by 1-log greater DNA quantity detected in 95 °C heated cysts (−0.1 log fg) than trophozoites (−1.2 log fg) (black bars, Fig. 4a, c). However, when using the excystation assay to evaluate the cyst survival following heat treatment, cysticidal temperature was documented as 65 °C (10 min) for cysts of *A. castellanii*, *A. polyphaga*, and six *Acanthamoeba* isolates from hospital and river water (Coulon et al. 2010). The inconsistency in cysticidal temperature between this and previous studies may be due to different *Acanthamoeba* strains tested and/or the disparity in analytical assay, as cross-linking of cyst external structures may impair excystation even if the cysts are viable (Coulon et al. 2010) and detectable by EMA-qPCR and qPCR.

In conclusion, EMA-qPCR shows promise as a rapid, sensitive, and reliable assay for quantifying viable *Acanthamoeba* and may be used as an alternative to time-consuming culture/MPN methods. By treating samples with and without EMA and analyzing by qPCR, this study provides viable and total *Acanthamoeba* densities in various kinds of aquatic environments along with the values of viability ratio (0.11–0.94), which are valuable information to better understand the relative risk for human exposed to *Acanthamoeba*. Moreover, reductions in both EMA-qPCR and qPCR detectable *Acanthamoeba* target sequences observed in superheat testing indicate that heating *A. castellanii* at 75–95 °C compromised the integrity of amoebic cell wall, cytoplasmic membrane and DNA, and trophozoites were much more sensitive to superheating than cysts and cells at transient stage.

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