Evaluation of DNA extraction methods and dilution treatment for detection and quantification of *Acanthamoeba* in water and biofilm by real-time PCR

Ching-Wen Chang and Ying-Chieh Wu

**ABSTRACT**

*Acanthamoeba*, human pathogens and natural hosts of pathogenic bacteria, may be accurately detected and quantified by real-time PCR if *Acanthamoeba* DNA are properly extracted and PCR inhibitors are effectively eliminated. However, the optimization of DNA extraction methods has not been reported for *Acanthamoeba*. This study compared the effectiveness of two DNA extraction/purification methods (FastDNA® Spin Kit for soil and Wizard® SV genomic DNA Purification System) by using trophozoites and cysts of *Acanthamoeba castellanii* and water and biofilm samples of cooling towers. DNA of *A. castellanii* extracted with the FastDNA® Kit and quantified by TaqMan PCR resulted in a lower variation (CV of Ct, 3%), greater linearity ($R^2 = 0.99$), and higher slopes (1.177–1.187 log fg DNA/log cell number) as compared to that by the Wizard® Kit. For field testing, the number of *Acanthamoeba*-positive samples and the *Acanthamoeba* DNA quantity were both greater with the FastDNA® Kit than with the Wizard® Kit ($P = 0.016$ and $<0.0001$, respectively). Beneficial effects with dilutions of extracted DNA were also revealed with the FastDNA® Kit ($P = 0.0003$). In conclusion, DNA extraction by the FastDNA® Kit coupled with dilution of extracted DNA and PCR analysis are recommended for detecting and quantifying environmental *Acanthamoeba*.

**Key words** | *Acanthamoeba*, biofilm, real-time PCR, water

**INTRODUCTION**

*Acanthamoeba* are free-living amoebae (FLA) found worldwide (Mergeryan 1991) in two distinct physiological stages: metabolically active trophozoite and resting cyst. They have been isolated from many countries, e.g. rivers, soils, and lakes in Bulgaria (Tsvetkova et al. 2004), tap water sources in Japan (Edagawa et al. 2009), cooling towers in Belgium (Behets et al. 2007), thermal spas in Spain (Penas-Ares et al. 1994), domestic water in Korea (Jeong et al. 2007) and Hong Kong (Booton et al. 2002), and spring recreational area in Taiwan (Hsu et al. 2009) and Switzerland (Gianinazzi et al. 2009). Human exposure to *Acanthamoeba* may cause invariably lethal granulomatous amoebic encephalitis and severe infection of the cornea. In addition to their pathogenic characteristic, *Acanthamoeba castellanii* are natural hosts of aquatic pathogens, e.g. *Helicobacter pylori* and *Legionella pneumophila* (Greub & Raoult 2004). These pathogens, collectively named amoeba-resistant bacteria (ARB), resist to amoebic phagocytosis and replicate within *Acanthamoeba* (Greub & Raoult 2004). Following amoebic lysis, a substantial quantity of pathogenic ARB is released into aquatic environments, posing infectious risk to human.

With consideration of *Acanthamoeba* as human pathogens and ARB reservoirs, it is essential to quantify *Acanthamoeba* particularly in the niches where both acanthamoebae and ARB are present, e.g. cooling towers (Declerck et al. 2007). However, the information on quantitative data is very limited (Penas-Ares et al. 1994; Behets et al. 2007). This is partly attributed to the complexity...
nature of the conventional quantification method with serial dilution of samples, amoebic culture seeded with bacteria, and subsequent most-probable-number (MPN) calculations (Rodriguez-Zaragoza 1994; Behets et al. 2007). Such MPN method is time-consuming, labor-intensive, and incapable of identifying amoebae failed to grow at given culture condition (Riviere et al. 2006). On the other hand, molecular analysis of nucleic acids using real-time quantitative polymerase chain reaction (real-time qPCR) may reduce time demand, detect non-culturabale cells, and quantify Acanthamoeba by personnel without excellent expertise in recognizing morphological features of acanthamoebae. However, to quantify environmental Acanthamoeba by real-time qPCR, it should be noted that biochemical components present in environmental substrates (Wilson 1997) may affect the efficiencies of DNA extraction methods (Lloyd-Jones & Hunter 2001). Besides, PCR inhibitors rich in field samples, such as humic substances and fulvic acid, could be coextracted with DNA and interfere with subsequent PCR amplification (Wilson 1997). As the accuracy of microbial quantification by real-time qPCR is mainly dependent on the quality and quantity of extracted DNA, the interferences of environmental biochemical substances and PCR inhibitors on the efficiencies of DNA extraction and PCR amplification should be concerned. Nevertheless, to our knowledge, the optimization of DNA extraction methods has not been reported for Acanthamoeba.

In this study, the performance of two commercial DNA extraction methods, i.e. FastDNA Spin Kit for soil and Wizard® SV genomic DNA Purification System, were evaluated with trophozoites and cysts of A. castellanii. Both of the extraction methods have been applied to environmental samples (Kuiper et al. 2006; Novikova et al. 2006) and recently used in protozoan cells (Jiang et al. 2005; Kuiper et al. 2006; Riviere et al. 2006; Ofer et al. 2008); however, their extraction efficiencies have not been simultaneously assessed yet. In addition to testing with A. castellanii, biofilms and water samples were also collected from cooling towers to evaluate the applicability of these two DNA extraction methods based on the number of Acanthamoeba-positive samples and the PCR-determined DNA quantity of Acanthamoeba. The effectiveness of anti-PCR inhibition by DNA dilution treatment was also assessed.

**METHODS**

**Strains of Acanthamoeba and culture conditions**

A. castellanii (ATCC 30234) trophozoites were axenically cultivated at 25°C for 3 days in proteose-yeast-glucose medium (ATCC 712 medium). As for cysts, 3-day amoebic cultures were harvested by centrifugation at 200 x g for 8 min. The cell pellet was resuspended in 10 ml of encystment medium (0.1 M KCl, 0.02 M Tris-HCl, 8 mM MgSO4, 0.4 mM CaCl2, 1 mM NaHCO3), followed by incubation at 25°C for 10 days (Riviere et al. 2006). No cyst in proteose-yeast-glucose medium and no trophozoite in the encystment medium were confirmed by microscopically examining all fields of the sample transferred onto a hemocytometer (Marienfeld, Germany).

**Cell and DNA dilution series**

Trophozoites and cysts of A. castellanii were centrifuged at 200 x g for 8 min and at 2,000 x g for 5 min, respectively. Cell pellets were resuspended in 2 ml sterile Page’s Amoeba Saline (PAS; 120 mg NaCl, 4 mg MgSO4·7H2O, 4 mg CaCl2, 142 mg Na2HPO4, and 136 mg KH2PO4 in 1 L ultrapure water), counted by a hemocytometer (Marienfeld), and serially diluted with PAS. An aliquot (100 µl) of trophozoites and cysts from each of the dilutions was added to 200 ml PAS, followed by filtration over a RTTP Isopore membrane (1.2 µm pore size, Millipore, USA) in a vacuum < 0.3 x 105 Pa to obtain 3 – 2.5 x 106 cells/filter. The filtration step added was due to the consideration of environmental water samples that might contain a low amount of cells. In addition to these cell-based diluted samples, serial dilutions of the DNA extracted from the filters containing 2.5 x 106 cells were also prepared (DNA extraction assays indicated below).

**DNA extraction**

FastDNA Spin Kit for soil (MP) (MP Biomedicals, USA) disrupts the cells by bead beating method prior to isolation and purification of DNA, whereas Wizard® SV Genomic DNA Purification System (Promega) (Promega Corporation, USA) adopted chemical lysis to release DNA.
After cutting filters in pieces and transferring all of the pieces into a 2-ml tube, DNA extraction by MP was performed according to the manufacturer’s instructions, except for extending the bead-beating time in FastPrep® Instrument (MP Biomedical) from 30 s to 1 min and increasing the volume of DNA elution solution (DES, DNase/pyrogen free water) from 50 μl to 100 μl.

DNA extracted by Promega was performed following the methods of Riviere et al. (2006) and the manufacturer’s protocol in which the DNA could be purified by either a vacuum or a microcentrifuge method. To select an optimal purification assay with Promega kit, 6 log A. castellanii trophozoites filtered on cellulose ester membranes (0.8 μm pore size, Millipore) were lysed by vigorously stirring the cut filters with 8 ml of 4 M filtered guanidium thiocyanate for 30 s. The lysates were extracted and purified by a microcentrifuge or a vacuum manifold following the manufacturer’s instruction with modification of the volume of Nuclease-Free water (200 μl) for DNA elution (Riviere et al. 2006). The extracted DNA was quantified with a spectrophotometer (Shimadzu, Japan) and analyzed by real-time qPCR described below. We observed that DNA purified by a microcentrifuge resulted in higher concentration of extracted DNA (53.5 m g/ml) and lower mean value of cycle threshold (Ct) (13.3) by real-time qPCR compared to that of the vacuum method (22 m g/ml and 15.5, respectively) (data not shown). Thus, the microcentrifuge method was adopted in this study.

DNA purity

DNA extracted by MP and Promega from the filters containing 2.5 × 10⁶ trophozoites or cysts of A. castellanii were diluted with TE buffer. The absorbance recorded at 260 nm and 280 nm by a spectrophotometer (Shimadzu) was used to calculate an A₂₆₀/A₂₈₀ ratio, an indicator of DNA purity (Kim et al. 2005). The experiments were performed in triplicate.

Real-time qPCR

Sequences of the primers and TaqMan probe designed by Riviere et al. (2006) were used to amplify a fragment of approximately 66 bp on 18S rRNA gene as these primers/probe have been shown to successfully quantify DNA of A. castellanii extracted by Promega (Riviere et al. 2006) The reaction mix of PCR contained 10 μl LightCycler FastStart DNA Master Hybridization Probes mix (Roche Diagnostics, Germany), 240 nM forward primer, 240 nM reverse primer, 240 nM probe, and 10 μl extracted or standard DNA in 25 μl total reaction volume. The reaction mix was placed in LightCycler® 480 multiwell plate 96 (Roche Diagnostics) and centrifuged at 1,500 × g for 2 min at 4°C. After denaturation of DNA and activation of polymerase at 95°C for 10 min on LightCycler 480 (Roche Diagnostics), 45 cycles of amplification were conducted as 15 s at 95°C and 1 min at 60°C, followed by a final cooling step at 40°C for 15 sec. All PCR runs consisted of test samples, serially diluted DNA standards (0.1–10⁶ fg/μl), and non-template control (NTC). The DNA standard that consisted of plasmid yT&A vector (Yeastern Biotech, Taipei, Taiwan) carrying an PCR product from a fragment of A. castellanii (ATCC 30234) DNA amplified using the primers and PCR as described above, was synthesized by Mission Biotech Co., Ltd. (Taipei, Taiwan).

A standard curve of Ct values was constructed from DNA standards. The slope of the standard curve was adopted to calculate PCR amplification efficiency (E) according to the equation of $E = 10^{(-1/\text{slope})} - 1$ (Riviere et al. 2006). The DNA concentrations (fg/μl) of test samples were then determined by interpolation from the standard curve of Ct values. DNA quantity (fg) was calculated as DNA concentration multiplied by final elution volume (i.e. 100 μl and 202 μl for MP and Promega, respectively). The standard curves were further constructed from the dilutions of A. castellanii cells as “log DNA quantity vs log cell number”. The $R^2$ value was determined from the standard curves and the detection limit was regarded as the lowest cell count at $R^2 \geq 0.99$.

Cooling tower samples

Water, substrate-associated biofilm (SB) from the surface of water basins, and floating biofilm (FB) at liquid-air interface were collected from three cooling towers primarily following the sampling methods described by Declerck et al. (2007). One litre of water sample was then filtered over a 1.2-μm RTTP Isopore membrane (Millipore) in a
vacuum \(\equiv 0.3 \times 10^5\) Pa. FB-associated cells collected on collodion-coated microscope slides (14.3 cm\(^2\)) were detached by a sterile cell scraper in 45 ml of phosphate buffer saline, which were vortexed for 1 min and filtered over 0.4 \(\mu\)m HTTP Isopore membranes (Millipore). DNA of the filter-retained cells were then extracted by MP and Promega as described above.

SB samples were centrifuged at 3,000 \(\times g\) for 15 min and approximately 0.3 g of the pellet was transferred to a 1.5-ml centrifuge tube (Bottger, Germany). DNA in SB pellets were extracted as described above in “DNA extraction” with the modifications of cell lysis procedures: For Promega, instead of filtering and vortexing once in 8 ml of 4 M filtered guanidium thiocyanate for 30 s, SB pellets were vigorously vortexed for 1 min in 0.5 ml of 4 M guanidium thiocyanate, which were vigorously vortexed again for 30 s after supplement of 7.5 ml of guanidium thiocyanate. As for MP, instead of direct addition of 978 \(\mu\)l sodium phosphate buffer into a cell-contained Lysing matrix E tube as a first step according to the manufacturer’s instruction, SB pellets were vigorously vortexed in 500 \(\mu\)l sodium phosphate buffer for 1 min, followed by transferring the cell suspensions into a Lysing matrix E tube containing 478 \(\mu\)l sodium phosphate buffer.

**DNA purity**

The DNA purity of *A. castellanii* retained on filters and extracted by MP, presented as the mean (± SD) of \(A_{260}/A_{280}\) ratio, was 1.79 (± 0.04) and 1.72 (± 0.05) for trophozoites and cysts, respectively. The respective DNA purity extracted by Promega was 1.86 (± 0.05) and 1.97 (± 0.02) (data not shown). Kim et al. (2005) reported that \(A_{260}/A_{280}\) value for purified DNA lies between 1.8 and 2. Clark & Christopher (2000) indicated \(A_{260}/A_{280}\) ratio for pure DNA is near 1.8. Therefore, except for MP-extracted cysts, it appears that no significant amounts of RNA or proteins existed in the DNA extracted by MP or Promega.

**DNA dilution treatment**

DNA extracted from cooling tower samples by MP and Promega methods were serially diluted with TE buffer (i.e. 1:10, 1:100, and 1:200 for water samples; 1:10, 1:100, 1:200, 1:300 for SB; and 1:10 and 1:100 for FB). Diluted and undiluted DNA were amplified by real-time qPCR along with DNA standards and NTC. The environmental sample with a \(C\) value < 40 was considered as PCR-positive. The number of PCR-positive samples was recorded for each dilution. The quantity of *Acanthamoeba* DNA in PCR-positive sample was also determined based on DNA concentration, DNA elution volume, and dilution factor.

**Statistical analysis**

\(R^2\) and equations of standard curves and coefficient of variation (CV, %) of \(C\) values were determined by Microsoft Office Excel 2007 (Microsoft, USA). The nonparametric Wilcoxon signed rank test was conducted by SAS software version 9.1 (SAS, USA) to examine the differences of the performance between MP and Promega methods and the effects of DNA dilution on *Acanthamoeba* quantification. Statistical significance was considered \(P < 0.05\).

**RESULTS AND DISCUSSION**

**DNA purity**

The DNA purity of *A. castellanii* retained on filters and extracted by MP, presented as the mean (± SD) of \(A_{260}/A_{280}\) ratio, was 1.79 (± 0.04) and 1.72 (± 0.05) for trophozoites and cysts, respectively. The respective DNA purity extracted by Promega was 1.86 (± 0.05) and 1.97 (± 0.02) (data not shown). Kim et al. (2005) reported that \(A_{260}/A_{280}\) value for purified DNA lies between 1.8 and 2. Clark & Christopher (2000) indicated \(A_{260}/A_{280}\) ratio for pure DNA is near 1.8. Therefore, except for MP-extracted cysts, it appears that no significant amounts of RNA or proteins existed in the DNA extracted by MP or Promega.

**PCR amplification efficiency (E) and standard curve**

Figure 1 shows the standard curve of known *A. castellanii* DNA concentration and \(C\) value by real-time qPCR. The standard curve was linear for over 8 orders of magnitude, ranging from 0.1 fg/\(\mu\)l to 1 ng/\(\mu\)l, with a \(R^2\) value of 1 and a slope of – 3.51. The CV of \(C\) values in six replicates ranged between 1.2 and 3.7\%, with an E of 0.93.
As for DNA-based standard curves constructed from the dilutions of DNA extracted from filter-retained cells (Figure 2), $C_t$ of Promega-extracted DNA were constantly greater than those with MP-extracted DNA with a statistical significance ($P$, 0.0001) by approximately 2 units. The slopes of both standard curves were similar ($-3.418$ for Promega and $-3.411$ for MP) with the same $E$ value of 0.96 and $R^2$ of 1. The CV of $C_t$ in triplicate tests were 0.3–0.8% (MP) and 0.4–0.9% (Promega).

The excellent linearity ($R^2 = 1$), great PCR amplification efficiency ($E \geq 0.93$), and low CV (0.3–3.7%) revealed in Figures 1 and 2 indicated that the real-time qPCR assay responds accurately and precisely to DNA loads. However, the $C_t$ of MP-extracted DNA was significantly less than those of Promega-extracted DNA, suggesting that a higher DNA concentration, approximately four folds, was yielded by MP. After adjusting for final DNA elution volume (i.e. 100 $\mu$l for MP and 202 $\mu$l for Promega), Acanthamoeba DNA quantity yielded from MP/qPCR was still greater than that by Promega/qPCR. Low $C_t$ value with MP extraction method was also reported by Klerks et al. (2006), who observed the MP (previously named as Qbiogene, Bio101 extraction kit) produced the lowest mean $C_t$ value of TaqMan PCR on DNA of Salmonella enterica seeded to manure among five commercially available DNA extraction methods.

**Cell-based standard curve**

The standard curves of DNA quantity and number of A. castellanii ($3\times10^6$ cells) diluted on cell basis are presented in Figure 3. For trophozoites (Figure 3(A)), a good linearity ($R^2 = 0.99$) was observed for MP-extracted samples, whereas DNA quantity was not perfectly linear with cell number in Promega-treated ones ($R^2 = 0.94$). CV and the slope of standard curve for MP- and Promega-extracted trophozoites were 0.5–2.9% and 1.177 (MP) and 0.6–5.9% and 0.88 (Promega), respectively. For cysts (Figure 3(B)), a good linearity ($R^2 = 0.99$) was revealed regardless of the extraction methods used. CV and the slope were 0.5–2.2% and 1.187 for MP-extracted cysts and 0.5–4.6% and 0.981 for Promega-treated ones, respectively. Lower CV (3%), greater $R^2$ (0.99), and higher slopes (1.177–1.187) in MP-extracted A. castellanii as compared to those by Promega (i.e. <6%, 0.94–0.99, and 0.88–0.98, respectively) indicated that MP/qPCR may better quantify A. castellanii than Promega/qPCR in terms of reproducibility, accuracy, and sensitivity over 6 orders
of magnitude. The detection limit of MP/qPCR, considered as the minimum number of cells in a sample that can be detected and accurately quantified by real-time qPCR after the procedures of sample pretreatment and DNA extraction, was 3 cells/sample as a great linearity ($R^2 = 0.99$) between $3 \times 10^6$–$2.5 \times 10^6$ cells. Statistical analysis of trophozoite and cyst data further revealed that the DNA quantity determined by MP/qPCR assays was significantly different from that by Promega/qPCR ($P = 0.039$).

The nonlinearity with Promega/qPCR (Figure 3(A)) was mainly due to the deviation of DNA quantities measured in the samples containing 3 and 30 trophozoites from the linear expectation. In fact, a linear relationship ($R^2 = 0.995$) was observed with Promega/qPCR for samples containing $3 \times 10^7$–$2.5 \times 10^6$ trophozoites. Similar result could also be found in Riviere et al. (2006) study: According to their linear equation established at high cell counts ($10^3$–$10^6$ trophozoites), the $Ct$ value for the sample containing 10 trophozoites would expect to be 33.8; however, the measured mean $Ct$ value was around 31 (Riviere et al. 2006). Their result and our finding support the presence of nonlinearity for trophozoites extracted by Promega kit. However, the reason for this nonlinearity occurred at low trophozoite counts remains unsolved; future investigation is warranted.

According to the linear equations of MP-extracted samples shown in Figure 3, the DNA quantity of trophozoites was approximately twice that of cysts, in agreement with the finding by Riviere et al. (2006). This could be due to the variation in DNA content during the growth cycle of protozoa (Galluzzi et al. 2004) or different extraction efficiencies between trophozoites and cysts.

**Acanthamoeba in cooling tower samples**

Table 1 shows that the number of *Acanthamoeba*-positive samples was greater in MP-extracted ones than in those with Promega for water (9/12 vs 4/12) and SB samples (10/15 vs 5/15), whereas they were the same (5/9) for FB samples. For each of the DNA dilution folds, the number of PCR-positive samples by MP extraction was also greater than or equal to that by Promega in water and SB samples. In overall, the detection rate of *Acanthamoeba* by MP was statistically higher than that by Promega ($P = 0.016$).

<table>
<thead>
<tr>
<th>Sample type</th>
<th>No. of PCR positive†/no. of cooling tower samples</th>
<th>Dilution of extracted DNA samples</th>
<th>MP</th>
<th>Promega</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1 (undiluted)</td>
<td>2/3</td>
<td>1/3</td>
<td></td>
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<tr>
<td></td>
<td>10</td>
<td>3/3</td>
<td>2/3</td>
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<td></td>
<td>100</td>
<td>3/3</td>
<td>1/3</td>
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<td></td>
<td>200</td>
<td>1/3</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td>SB</td>
<td>1 (undiluted)</td>
<td>2/3</td>
<td>2/3</td>
<td></td>
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<tr>
<td></td>
<td>10</td>
<td>2/3</td>
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<td>3/3</td>
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<td>FB</td>
<td>1 (undiluted)</td>
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<td>10</td>
<td>1/3</td>
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<td></td>
<td>100</td>
<td>2/3</td>
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</table>

†Sample with a $Ct$ value $< 40$ was considered as PCR positive.

Table 1 | Number of *Acanthamoeba*-positive cooling tower samples determined by real-time qPCR following DNA extraction using MP or Promega method and dilution treatment

For PCR-positive water samples (Figure 4(A)), the mean quantity of *Acanthamoeba* DNA determined by MP/qPCR was greater than that by Promega/qPCR regardless of DNA dilution folds. For SB (Figure 4(B)) and FB (Figure 4(C)), MP-extracted and diluted samples also resulted in a higher mean of *Acanthamoeba* DNA quantity than those extracted by Promega. Results of statistical analysis on cooling tower data showed that DNA quantity of *Acanthamoeba* determined by MP/qPCR was statistically greater than that by Promega/qPCR ($P < 0.0001$), particularly in water ($P = 0.02$) and SB samples ($P = 0.016$).

Results shown in Table 1 and Figure 4 demonstrated a significantly higher detection and greater DNA quantity of *Acanthamoeba* by MP/qPCR as compared to Promega/qPCR. DNA-based dilution of *A. castellanii* cells (Figure 2) also indicated a greater DNA quantity by MP/qPCR than by Promega/qPCR. These consistent findings suggest that MP method possesses a better DNA extraction efficiency than Promega for *Acanthamoeba*. MP kit extracts DNA by bead beating in combination with chemical lysis buffers, while Promega extraction is solely based on...
chemical lysis. The better performance with MP/qPCR could be partly due to the greater DNA yields obtained by bead beating with subsequent lysis than by chemical lysis alone. The bead beating method has been shown to improve the extraction efficiency of DNA from bacteria of the Mycobacterium tuberculosis complex (Amaro et al. 2008). Two recent investigations also showed that MP/qPCR resulted in the lowest mean Ct value on DNA of S. enterica seeded to manure (Klerks et al. 2006) and the highest copy number of Mycobacterium paratuberculosis (Cook & Britt 2007) among various DNA extraction methods. Greater positive detection rates by MP/qPCR were also reported by Jiang et al. (2005) in their evaluation of DNA extraction methods for detection of protozoan Cryptosporidium in wastewater and storm water. These previous findings accord with our results that MP/qPCR performs better in detection and quantification of targeted microorganisms.

**Dilution effects on anti-PCR inhibition**

Figure 4 also shows that the mean of DNA quantity was increased in MP-extracted and diluted samples by 0.9–2.2 log fg, 0.5–1.5 log fg, and 0.9–1.2 log fg for water, SB and FB, respectively, as compared to undiluted ones. For Promega-extracted samples, the respective increases in mean DNA quantity were 0.5–0.9 log fg, 0.2–0.7 log fg, and 0.2–0.5 log fg, which were less than those by MP. The dilution treatment significantly increased the DNA quantity of Acanthamoeba determined by MP/qPCR ($P = 0.003$), whereas no statistical significance was observed for Promega/qPCR-analyzed ones.

The finding that less DNA quantities were measured in undiluted DNA than diluted DNA indicated that water and biofilms in cooling towers were rich in PCR inhibitors, e.g. humic substances known as the most commonly reported inhibitors in water, sediments, and soils (Wilson 1997). Some of PCR inhibitors were coextracted with DNA and interfered with PCR amplification, leading to an underestimation of Acanthamoeba DNA load. Dilution of the extracted DNA successfully reduced or eliminated the impact of PCR inhibitors, and thereby significantly improved PCR amplification efficiency. According to the results with MP/qPCR assay, cooling tower samples had to be diluted at least tenfold to improve PCR amplification. Instead of this rapid and straightforward treatment, further purification of MP-extracted DNA may also relieve PCR inhibition by gel chromatography (Arbeli & Fuentes 2007) or by agarose gel electrophoresis followed by agarase digestion (LaMontagne et al. 2002). However, the purification procedure is time-consuming and might cause DNA loss.

To estimate Acanthamoeba load in cooling water samples, the standard curve constructed with trophozoites...
CONCLUSIONS

Accurate detection and quantification of environmental *Acanthamoeba* may be achieved by real-time qPCR if the DNA is efficiently extracted to be a representative of the environment from which it was obtained and is free of PCR inhibitors that may bias the PCR data. Selection of an appropriate DNA extraction method is one of the keys to obtaining a representative DNA sample, reducing the levels of PCR inhibition, and maximizing the sensitivity of real-time qPCR assay. This study demonstrates that the cooling tower is believed to facilitate the growth of protozoa (*Pagnier et al. 2009*). The greatest log DNA quantity determined by MP/qPCR among undiluted and diluted DNA was input as the Y value of the standard curve for each of samples. The resulting X value was then anti-log transformed to determine *Acanthamoeba* count. As a result, *Acanthamoeba* were ranged between 118 and 4,211 cells/L with a mean of 1,495 cells/L. Concentrations of thermophilic *Acanthamoeba* determined by the MPN method with culturing at 44°C were reported between 0 and 452 cells/L for cooling water at Belgian electrical power plants (*Behets et al. 2007*). The difference in *Acanthamoeba* concentration between the present and previous studies might be attributed to the differences in analytical methods and water characteristics, e.g. concentration of bacteria as amoebic food supply.

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REFERENCES


(Y = 1.177X – 0.209, Figure 3(A)) was adopted due to the consideration that the cooling tower is believed to facilitate the growth of protozoa (*Pagnier et al. 2009*). The greatest log DNA quantity determined by MP/qPCR among undiluted and diluted DNA was input as the Y value of the standard curve for each of samples. The resulting X value was then anti-log transformed to determine *Acanthamoeba* count. As a result, *Acanthamoeba* were ranged between 118 and 4,211 cells/L with a mean of 1,495 cells/L. Concentrations of thermophilic *Acanthamoeba* determined by the MPN method with culturing at 44°C were reported between 0 and 452 cells/L for cooling water at Belgian electrical power plants (*Behets et al. 2007*). The difference in *Acanthamoeba* concentration between the present and previous studies might be attributed to the differences in analytical methods and water characteristics, e.g. concentration of bacteria as amoebic food supply.


