Quantitative real-time PCR does not reliably detect single fecal indicator bacteria in drinking water

Tamara Krapf, Roger Michael Kuhn, Peter Kauf,
Corinne Helene Gantenbein-Demarchi and Lars Fieseler

ABSTRACT

The microbial quality of drinking and environmental water is usually determined by culture-based detection of fecal indicator bacteria according to ISO reference methods 16649-1 and 7899-2, respectively. Because of an increasing demand for rapid, culture-independent methods, we tested three quantitative polymerase chain reaction (qPCR) approaches for the simultaneous detection of both, *Escherichia coli* and *Enterococcus* spp., using either 16S rRNA or 16S rDNA as a target molecule. Filter sterilized drinking water was artificially contaminated with bacteria from either high or low nutrient culture conditions and directly analyzed after membrane filtration without any other enrichment. Depending on the culture condition used, qPCR analyses revealed a lower limit of detection of 1–10 *E. coli*/100 ml and 10–100 *E. faecalis*/100 ml, respectively. In addition, the microbial quality of different surface water samples was monitored. The analyses revealed a clear correlation between viable cell counts and qPCR data. However, the safe and reliable detection of 1 CFU/100 ml failed.

Key words | drinking water, *E. coli*, *Enterococcus faecalis*, qPCR

Tamara Krapf
Roger Michael Kuhn
Corinne Helene Gantenbein-Demarchi
Lars Fieseler (corresponding author)
ZHAW, Zurich University of Applied Sciences, Institute of Food and Beverage Innovation,
Wädenswil 8820,
Switzerland
E-mail: *lars.fieseler@zhaw.ch*

Peter Kauf
ZHAW, Zurich University of Applied Sciences, Institute of Applied Simulation,
Wädenswil 8820,
Switzerland

Roger Michael Kuhn
Present address: Department of Foundation Studies in Science,
ZHAW, Zurich University of Applied Sciences,
Wädenswil 8820,
Switzerland

INTRODUCTION

Fecal contaminated drinking water can cause diarrhea if pathogens are ingested. Worldwide, over 80% of cases of diarrhea are associated with unsafe drinking water, lack of sanitation or lack of hygiene. This leads to 1.5 million deaths by diarrhea annually, particularly in developing countries (Prüss-Üstün *et al.* 2008). Water-borne diseases have also been reported in industrial nations, but to lesser extents (Maurer & Stürchler 2000; Hrudey *et al.* 2003). *Escherichia coli* and species of the fecal *Enterococcus* group (*Enterococcus* spp.) are the most important indicators of fecal drinking water contamination. Both must not be present in a 100 ml sample volume (Anonymous 1998; Anonymous 2005; WHO 2011). Presence of fecal contamination by *E. coli* in drinking water indicates that pathogenic bacteria may also be present in a sample. *E. coli* is considered as the best biological representative of (fecal) pathogens in drinking water, as it is present up to 94.1% in human feces and up to 92.6% in animal feces. It is a reliable biological drinking water indicator for public health protection (Edberg *et al.* 2000). Hence, even 1 CFU/100 ml indicates that pathogens might be present, the latter proposing a health risk. The microbial quality of natural bathing waters, i.e. rivers, ponds, and lakes, is defined by the same hygiene indicators. Admittance of bathing in such waters is based on health grounds according to the classification of four quality groups in response to the CFU counts of both *E. coli* and *Enterococcus* spp. in 100 ml (Schaffner *et al.* 2013).

The culture-based detection of *E. coli* and *Enterococcus* spp. by the ISO reference methods 16649-1 and 7899-2, respectively, requires membrane filtration of water samples and an incubation of 24–48 h to reliably detect a single

doi: 10.2166/ws.2016.052

viable bacterium in a sample volume of 100 ml. This method is referred to as 'the golden standard' in drinking water microbial analyses. However, a faster, but as sensitive and specific method as the reference method would be useful and molecular techniques, e.g. polymerase chain reaction (PCR), tend to meet these requirements today (Frahm & Obst 2003; Sen et al. 2011; Mendes Silva & Domingues 2015). Ribosomal RNA (rRNA) is seen as a cell viability indicator and was suggested as a promising target molecule for detecting living cells (Keer & Birch 2003). Compared to DNA the RNA features a restricted half-life and is less stable after cell death (Bustin & Nolan 2004). Both 16S rDNA and 16S rRNA served as a target for the detection and identification of bacteria from different environmental samples, although rDNA can also be amplified from dead organisms (Harwood et al. 2004; Ryu et al. 2013). Detection of 16S rRNA was applied for recreational water monitoring of both *E. coli* and *Enterococcus* spp. by reverse transcription (RT)-qPCR (Bergeron et al. 2011), while detection of 23S rRNA was applied to detect fecal indicators in rain water, surface waters or ambient marine and fresh recreational waters by quantitative PCR (qPCR) (Whitman et al. 2010; Ahmed et al. 2012; Anonymous 2015). The combination of viability dyes such as ethidium mono azide or propidium mono azide (PMA) and DNA amplification led to the development of the viability-PCR. The technique relies on the permeability and integrity of the cell membrane. The viability dye accumulates inside dead cells only and intercalates into the DNA. The intercalation of the dye into the DNA inhibits DNA amplification (Nogva et al. 2003; Nocker et al. 2006; Fittipaldi et al. 2012).

In their review article about the detection of microorganism in water by PCR methods Botes et al. (2013) concluded that standardized protocols and improvements in method validation are needed for qPCR-based microbial water analysis. In order to address these issues, this study intended to develop a culture-independent *Taq*Man® (hydrolysis probe)/qPCR-based protocol for the simultaneous detection of *E. coli* and *Enterococcus* spp., which is applied directly after membrane filtration using 16S rRNA or 16S rDNA as target molecules without any enrichment cultivation. The microbial quality of untreated drinking water and environmental samples was determined applying different qPCR approaches and the reference methods.

MATERIALS AND METHODS

Bacterial strains, growth conditions and sample preparation

E. coli (ATCC 25922) and *Enterococcus faecalis* (ATCC 19433) were used for the experiments. To simulate nutrient-rich culture conditions the bacteria were incubated in LB broth at 37 °C, centrifuged (5,000 × g, 5 min, 5 °C), washed twice, and resuspended in 0.9% (w/v) NaCl. For nutrient-limited conditions 2 ml of the overnight culture were centrifuged, washed twice, resuspended in 60 μl of LB-broth, and added to 60 ml of sterile deionized water (dH_2O). The suspension was further incubated under constant agitation at 25 °C and 50 rpm for 3 days. Bacteria were then harvested by centrifugation (9,450 × g, 5 min, 5 °C) and prepared as described above. The total cell count was determined using a counting chamber (Neubauer, depth: 0.01 mm, area: 0.0025 mm^2). Then filter sterilized (0.45 μm, VacuCap® 60, Pall) drinking water was artificially contaminated at concentrations of approx. 1, 10, 100, and 1,000 cells/100 ml. For viability-qPCR applications heat-treated bacteria (100 °C, 15 min) and non-inoculated water served as negative controls. Individual and independent experiments were repeated at least three times.

Culture-based analysis

Either 100 ml or 1,000 ml of artificially contaminated water were membrane filtered (0.45 μm, Ø 47 mm, Sartorius, Microsart CN Filter). Then the filter was transferred onto Tryptone Bile X-Glucuronide Agar (Biolife) for the detection of *E. coli* and onto Slanetz-Bartley Agar (Biolife) for the detection of *Enterococcus* spp. according to ISO 16649-1 and 7899-2, respectively. Tryptic-Soy-Agar (Biolife) was used for the revitalization of *E. coli*. Presumptive colonies of *Enterococcus* spp. isolated from natural water samples were confirmed on Bile-Esculin-Agar (Biolife).

Viability dyeing and crosslinking

Either 100 ml or 1,000 ml water samples were artificially contaminated with heat-treated and non-heat-treated bacteria and were membrane filtered (0.45 μm, Ø 47 mm, Sartorius, Microsart CN Filter). The filter membrane was then placed into a sterile petri dish (Ø 60 mm), covered with 1 ml of 0.9% (w/v) NaCl and 10 μl PMA (200 μM). In the non-treated control 10 μl 0.9% (w/v) NaCl was added. Incubation of immersed membranes (with and without PMA) was performed in a light-proof Styrofoam box covered with aluminum foil (30 min, 30 rpm, room temperature). Cross-linking was performed for 30 min and 30 rpm at room temperature using LED lamps (470 nm) positioned in a self-made lid box lined with aluminum foil inside.

DNA isolation

Either 100 ml or 1,000 ml of artificially contaminated water samples were membrane filtered (0.45 μm, Ø 47 mm, Sartorius, Microsart CN Filter). DNA was extracted applying the RapidWater® DNA Isolation Kit (MoBio) according to the manufacturer's recommendations with minor modifications. To eluate isolated DNA the spin filter was loaded with 30 μl of the elution buffer, incubated at 50 °C for 5 min (Thermomixer comfort, Eppendorf) and centrifuged (13,000 × g, 1 min). DNA elution was performed twice using the first eluate for the second elution.

RNA isolation and reverse transcription

Either 100 ml or 1,000 ml of the inoculated water samples were membrane filtrated using a 0.45 μm syringe filter unit (Chromafil CA-45/15 MS-S, Macherey Nagel) (Wohler *et al.* 2012). Briefly, acetone (AppliChem) and ambient air were aspired into a syringe and the syringe was attached to the syringe filter unit. The acetone was pressed through the syringe filter unit to dissolve the filter inside. The filtrate was collected in a tube containing 1 μl latex beads (Polysterene, Sigma Aldrich). The tube was briefly vortexed and centrifuged (8,000 × g, 10 min). The supernatant was removed and the tubes were dried for 10 s. RNA was then isolated using the RNeasy Protect Bacteria Mini Kit (Qiagen) following the manufacturer's standard protocol for enzymatic lysis, proteinase K digestion and mechanical disruption of bacteria with the following modifications: 15 μl Proteinase K (>600 mAU/ml; Qiagen) and 100 μl TE buffer (30 mM Tris–HCl, 1 mM EDTA, pH 8.0) containing 15 mg/ml Lysozyme (AppliChem) were added to the pellet and mixed by pipetting, followed by an incubation at 25 °C for 10 min. 350 μl RLT buffer (Qiagen) and 25 mg of acid-washed glass beads (Sigma-Aldrich, Ø 150–600 μm) were added and the cells were disrupted by shaking (1,400 rpm, 5 min) in a Thermomixer comfort (Eppendorf). 5 μl of carrier RNA (RNeasy Micro Kit; Qiagen) was added to the lysate, briefly vortexed until the pellet was re-suspended, and centrifuged (16,000 × g, 10 s). The supernatant was transferred into a new tube and 330 μl pure ethanol (AppliChem) was added. The suspension was transferred to an RNeasy micro spin column and centrifuged for 15 s at 8,000 × g. After discarding the flow through, RNA purification was carried out using the RNeasy Micro Kit (Qiagen), including on-spin DNase digestion and repeated elution with an additional 14 μl of water to receive a final eluate of 28 μl. Individual experiments were repeated 10 to 15 times for the 100 ml sample volume and at least three times for the 1,000 ml sample volume.

Reverse transcription (RT) was carried out with the first strand complementary DNA (cDNA) Synthesis Kit for RT-PCR (AMV) from Roche, using random primers according to the manufacturer's protocol but excluding gelatin and dCTP. RT was carried out in a thermocycler (TC-3000, Techne): incubation (25 °C, 10 min), RT (42 °C, 30 min) and denaturation (99 °C, 5 min).

Quantitative PCR

E. coli and *Enterococcus* spp. were detected by (RT-)qPCR using oligonucleotides targeting the 16S rRNA or rDNA, respectively, applying a LightCycler® 480II (Roche) and LightCycler® 480 Probes Master Kit (Roche). The reaction was carried out in a volume of 20 μl. The *E. coli* qPCR assay contained 10 μl of 2× LightCycler® 480 Probes Master Mix (Roche), 400 nM of each primer (forward: 5′-AGCGGGGAG GAAGGGAGTAAAG-3′; reverse: 5′-GACTCAAGCTTG CCAGTATCAGATG-3′), 200 nM of the corresponding locked nucleic acid probe (5′-FAM-CCTTTGCTC{A}TTG{A} CGTT{A}CCCGCAG{A}AG-BHQ1-3′) and 5 μl template. The *Enterococcus* spp. qPCR assay contained 10 μl of 2× LightCycler® 480 Probes Master Mix (Roche,), 500 nM of each primer (forward: 5′- ATGGAGGAACACCAGTGGCGAA G-3′, reverse: 5′-AGCACTGAAGGGCGGAAACCCTCC-3′), 200 nM of the corresponding probe (5′-YYE-CTCTGG TCTGTAACTGACGCTGAGGCTCG-BHQ1-3′) and 5 μl template per sample. Each sample was measured in duplicate. For the negative control, 5 μl PCR-grade water (Ambion) was used instead of a template, while 1 μl of genomic DNA of *E. coli* or *E. faecalis* served as positive controls. The qPCR conditions were as follows: pre-incubation (1 cycle) 10 min at 95 °C, amplification (45 cycles) 10 s at 95 °C, 30 s at 67 °C, 1 s at 72 °C. qPCR data were analyzed using the Light Cycler® 480 Software (Roche) and Abs Quant/2nd Derivative Max (High Sensitivity).

Calculation of DNA copy numbers

Genomic DNA of *E. coli* or *E. faecalis* was isolated applying the DNeasy Tissue and Blood Kit (Qiagen) according to the protocol for Gram-positive and Gram-negative bacteria. The DNA concentration was determined using a NanoDrop spectrometer (Thermo Scientific). Based on the size of the complete genome (*E. coli*: 4.7×10^6 bp; *E. faecalis*: 3.2×10^6 bp), the Avogadro's number (6.02×10^{23} mol^{-1}) and the assumption that the average weight of a base pair (bp) is 650 Daltons, copy number can be calculated according to the following equation: (amount [ng] * 6.022×10^{23})/(length [bp] * 1×10^9 * 650) (http://cels.uri.edu/gsc/cndna.html). Copy numbers were calculated based on the qPCR standard curve (*E. coli*: $y = -1.526 \ln(x) + 42.335$ ($R^2 = 0.9962$), *Enterococcus faecalis*: $y = -1.603 \ln(x) + 41.288$ ($R^2 = 0.9905$)) where x gives the copy numbers after C_t-value is applied for y.

Water sampling and analysis

Drinking water samples were collected before UV disinfection. Environmental water samples were taken from rivers, lakes, and natural ponds in the region of Zurich (Switzerland). All waters were sampled using sterile PET bottles containing 20 mg/l Sodium Thiosulfate (Huber Lab) and stored at 4 °C. Analysis of 100 ml sample volumes were carried out within 24 hours as described above. Additionally, the turbidity of environmental water samples was measured using a portable turbidimeter (Hach, 2100QiS) when v-qPCR was applied.

Statistical analysis

Statistical analysis was performed using the Kruskal-Wallis-test and non-parametric post-hoc analysis (pairwise Wilcoxon tests with Tukey HSD alpha correction) using the software package R (https://www.r-project.org/). The results were displayed in box-whisker plots showing median, upper and lower quartiles as well as upper and lower whiskers according to the standard implementation in R. Results were considered significant if the P-value was <0.05. The post hoc analysis was encoded in letters. All statistical tests were two-tailed.

RESULTS AND DISCUSSION

Targeting 16S rRNA by RT-qPCR revealed variable limits of detection

The analysis of 100 ml filter sterilized drinking water artificially inoculated with *E. coli* revealed variable lower limits of detection (LLOD) depending on the bacterial viability. The LLOD from nutrient-rich cultures was ≥10 cell equivalents (CE) *E. coli*/100 ml. It rose to ≥1,000 CE *E. coli*/100 ml if nutrient-limited culture conditions were applied. For *E. faecalis* the LLOD was ≥100 CE *E. faecalis*/100 ml from nutrient-rich cultures and ≥10 CE *E. faecalis*/100 ml from nutrient-limited cultures. This finding is partly in agreement with other studies (Bergeron *et al.* 2011). A tenfold increase in the sample volume to 1,000 ml resulted in a LLOD of ≥100 CE *E. coli*/100 ml from nutrient-rich cultures and ≥10 CE *E. coli*/100 ml from nutrient-limited cultures, respectively (Table 1, Figure S1, Supplementary material). The LLOD of *E. faecalis* was not altered (Table 1, Figure S2, Supplementary material). (Figures S1 and S2 are available with the online version of this paper.) In contrast, single contaminating bacteria, e.g. a minimum of 1 CFU/100 ml, could be reliably detected using the reference methods.

Bacterial viability is either defined by growth on a culture medium or by the expression of the rRNA operon (Oliver 2010). Because rRNA exhibits a relatively short half-time, it is applied as an indicator of bacterial viability (Smith & Osborn 2009). However, the efficiency of RNA extraction and RT varies depending on the extraction protocols and the priming strategy, resulting in varying yields of

Table 1 | Statistically estimated LLOD of fecal indicator bacteria in drinking water after application of different qPCR approaches

			Sample volume	
Method	**Organism**	**Cultivation**	**100 ml**	**1,000 ml**
qPCR	*E. coli*	Nutrient rich	10	1–10
		Nutrient limited	10	1–10
	E. faecalis	Nutrient rich	10	1–10
		Nutrient limited	10	1–10
RT-qPCR	*E. coli*	Nutrient rich	10	100
		Nutrient limited	1,000	10
	E. faecalis	Nutrient rich	100	100
		Nutrient limited	10	10
v-qPCR	*E. coli*	Nutrient rich	100[a]	100[a]
		Nutrient limited	1,000[a]	10[a]
	E. faecalis	Nutrient rich	100[a]	100[a]
		Nutrient limited	1,000[a]	100[a]

[a]Viable cells after PMA treatment.

cDNA, which is crucial for reliable quantification by RT-qPCR (Smith & Osborn 2009). Accordingly, targeting 16S rRNA for microbial water analysis revealed different LLOD depending on the cell's viability. The relative instability of the RNA molecules and the varying expression levels of the rRNA operon in combination with both, varying RNA and cDNA yields, may have resulted in inconsistent LLOD for the target microorganisms in different sample volumes. Hence, the detection of rRNA does not allow the detection of 1 *E. coli* or *E. faecalis*/100 ml making this target molecule less applicable for microbial water analysis.

Viability-qPCR approaches exhibited elevated limits of detection

Detection of *E. coli* and *E. faecalis* inoculated into filter sterilized drinking water by PMA treatment and qPCR, revealed a LLOD of ≥100 CE *E. coli* or *E. faecalis*/100 ml (nutrient-rich cultures) and of ≥1,000 CE *E. coli* or *E. faecalis*/100 ml (nutrient-limited cultures) (Table 1; Figures S3 and S4, Supplementary material, available with the online version of this paper). Analyzing a sample volume of 1,000 ml did not alter the LLOD of bacteria from nutrient-rich cultures, but a decrease of the LLOD was evident for bacteria from nutrient-limited cultures: ≥10 CE *E. coli*/100 ml and ≥100 CE *E. faecalis*/100 ml (Table 1). The control of non-heat treated bacteria without PMA treatment yielded the same LLOD as with PMA treatment confirming the detection of viable cells. As expected, the control of heat- and PMA-treated bacteria remained negative, confirming that PMA treatment inhibits qPCR of dead cells (Nocker & Camper 2009). In contrast, DNA of heat-treated bacteria without the addition of PMA was amplified. Although viability dyeing allowed differentiating between viable and dead cells, the LLOD determined by v-qPCR varied for the target organisms depending on the culture conditions, and was higher than in qPCR assays. This might be due to the filter immersion in NaCl-PMA-solution prior to DNA extraction and v-qPCR analyses. Because success of viability dyeing is dependent on different factors such as dye concentration, incubation time and period, and cell membrane integrity (Fittipaldi *et al.* 2012), the ratio of increased biomass and PMA concentration may not have been optimal to further decrease the LLOD. Again, a minimum of 1 CFU/100 ml could be reliably detected using the reference methods.

Detection of 1–10 CE *E. coli*/100 ml by 16S rDNA targeting qPCR

If 100 ml of filter sterilized drinking water were inoculated with *E. coli* or *E. faecalis*, the LLOD was ≥10 CE *E. coli* or *E. faecalis*/100 ml for bacteria from nutrient-rich and nutrient-limited cultures. Increasing the sample volume to 1,000 ml resulted in a LLOD of 1–10 CE *E.coli*/100 ml (Figure 1) and 1–10 CE *E. faecalis*/100 ml (Figure 2) from both culture conditions (Table 1). In comparison with the rRNA analysis, the LLOD's were lower in 100 ml sample volumes for each of the indicator bacteria and culture condition tested, likely because DNA is much more stable than rRNA (Bustin & Nolan 2004). Similar detection limits for rDNA driven approaches have been reported ranging from 1 to 27 CFU/100 ml for *Enterococcus* spp. and 2–25 CFU/100 ml for *E. coli* (Ahmed *et al.* 2012; Lam *et al.* 2014). These findings are in agreement with our data demonstrating that at least 10 CE *E. coli* or *E. faecalis* need to be present on the filter membrane for a reliable positive qPCR result regardless of the applied sample volume. Applying the reference methods a minimum of 1 CFU/100 ml was detected.

Detection of fecal indicator bacteria in water samples

The analysis of 54 drinking water samples revealed no or very low (<10 CFU per 100 ml) microbial contamination with

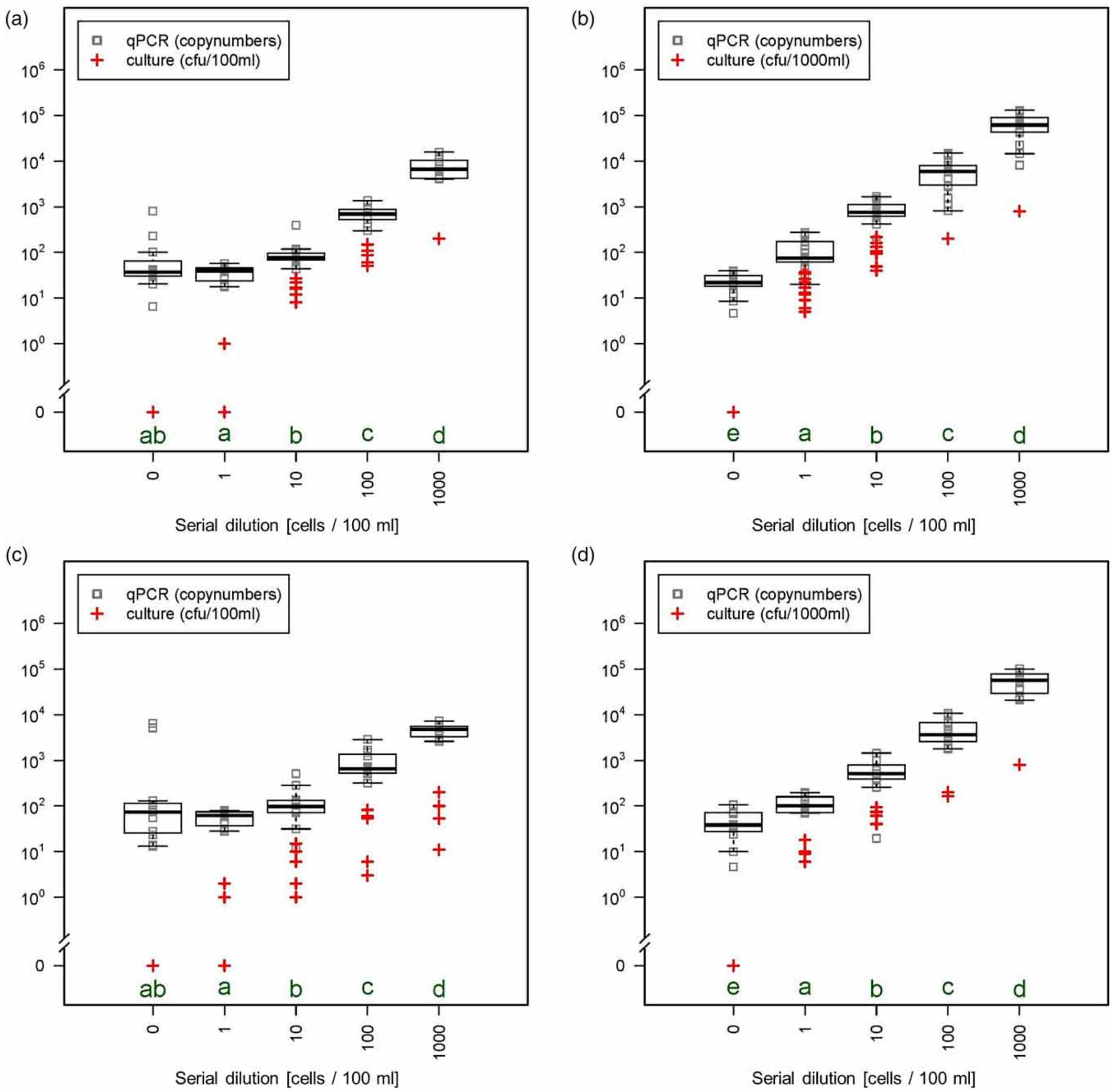

Figure 1 | Detection of *E. coli* in artificially contaminated drinking water by qPCR using 16S rDNA as a target molecule (squares) and by the reference method (crosses): (a) and (b) water was inoculated with bacteria from nutrient-rich cultures; (c) and (d) water was inoculated with bacteria from nutrient-limited cultures; (a) and (c) 100 ml sample volume; (b) and (d) 1,000 ml sample volume. Significant differences are indicated by letters a, b, c, d, and e.

fecal indicator bacteria. Only one sample was contaminated with 48 CFU of *E. coli* and 8 CFU of *Enterococcus* spp. per 100 ml sample volume. In this sample *E. coli* was also detected by qPCR, but *Enterococcus* spp. could not be detected (Figure S5, Supplementary material, available with the online version of this paper). This observation fits with our data indicating that at least 10 CE *E. coli* or *Enterococcus* spp. need to be present on the filter membrane for successful qPCR detection. The analyses of environmental surface water samples using culture and qPCR methods revealed comparable results for both *E. coli* and *Enterococcus* spp., with a relatively constant microbial load during the period of investigation (Figure S6, Supplementary material, available with the online version of this paper). In general, qPCR detected higher cell loads than the culture-based method, which is in agreement with other studies and probably due to the presence of dead or viable, but non-cultureable, (VBNC) cells (Ludwig & Schleifer 2000; Converse *et al.* 2012).

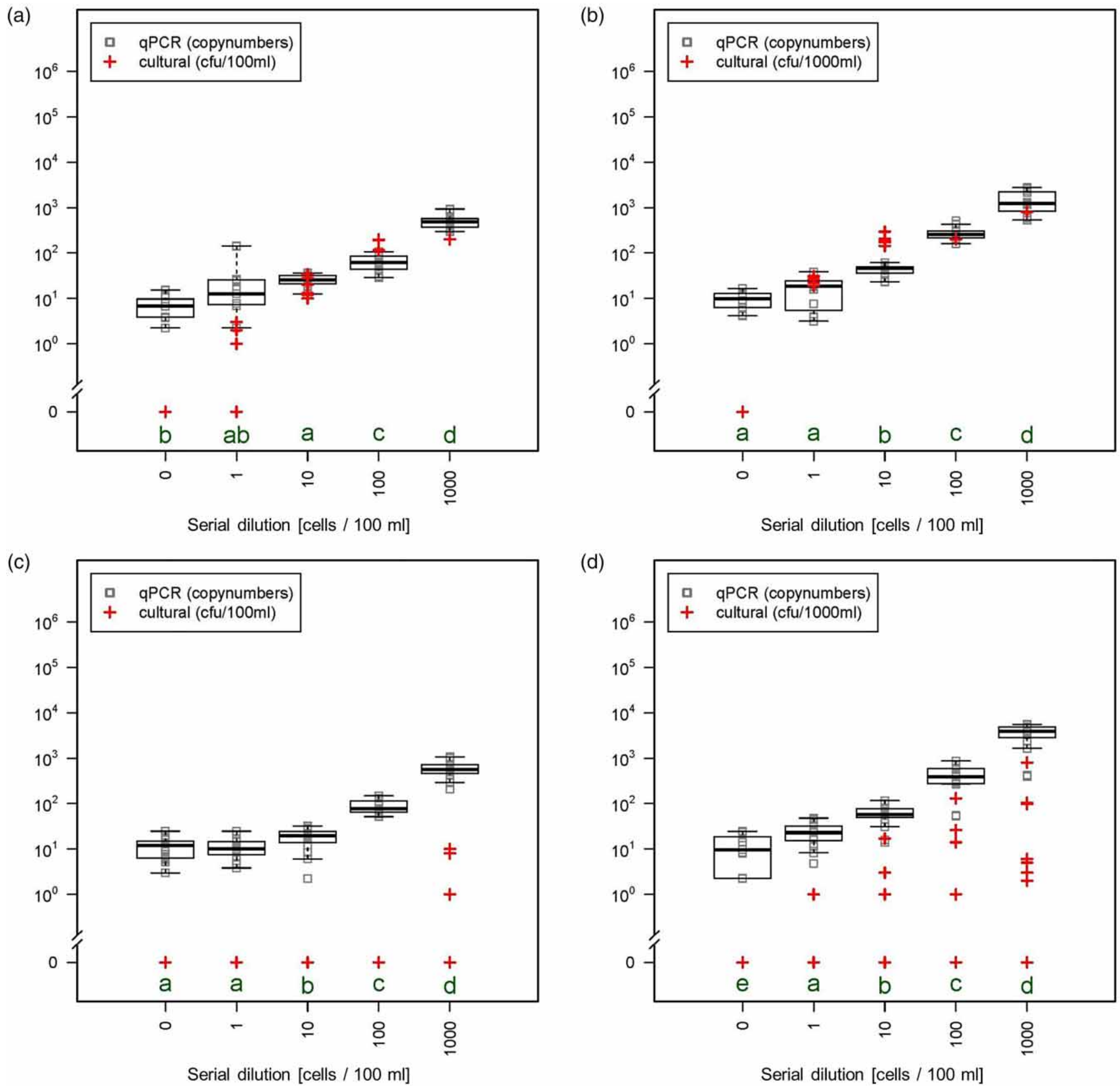

Figure 2 | Detection of *Enterococcus* spp. in artificially contaminated drinking water by qPCR using 16S rDNA as a target molecule (squares) and by the reference method (crosses): (a and b) Water was inoculated with bacteria from nutrient-rich cultures; (c and d) water was inoculated with bacteria from nutrient-limited cultures; (a and c) 100 ml sample volume; (b and d) 1,000 ml sample volume. Significant differences are indicated by letters a, b, c, d, and e.

Viability dyeing is not applicable for microorganisms in water, which was disinfected by UV light. False-negative results will be reported, because the cell membrane remains intact while the DNA is damaged (Nocker *et al.* 2007). Hence, in this study v-qPCR was applied to untreated environmental water samples with a turbidity <10 Nephelometric Turbidity Units (NTU). A turbidity >10 NTU negatively influences PMA treatment and detection by v-qPCR (Fittipaldi *et al.* 2012). Little or no difference in qPCR results were determined for samples treated with or without PMA which is in accordance with other studies (Varma *et al.* 2009). Only in a few cases qPCR results of PMA treated samples differed from PMA non-treated samples (Figure S7, Supplementary material, available with the online version of this paper). In these cases, the ratio between dead and viable cells was either >1,000, or dead cell numbers were $>10^4$ and viable cell counts $<10^3$. Such conditions significantly reduce the efficiency of PMA treatment (Fittipaldi *et al.* 2012). Because intact cells make up to 98% of the total cell counts in different waters (Berney *et al.* 2008), the results indicate that mainly viable cells were detected even in PMA non-treated samples.

CONCLUSIONS

Drinking water is tested positive for fecal contamination if 1 CFU *E. coli* or *Enterococcus* spp./100 ml is detected by the reference culture methods. However, culture-based approaches exclusively detect viable and culturable cells, but not VBNC cells. In contrast, PCR is able to detect not only viable, but also dead and VBNC cells (Oliver 2010) depending on the qPCR technique used and the gene target applied. This study compared three different qPCR approaches and demonstrates that detection of *E. coli* and *Enterococcus* spp. in drinking water can be performed in less than five hours using a single filtration without a pre-enrichment. Table 1 summarizes the LLOD for each qPCR technique applied. qPCR exhibited a LLOD of $\geq$10 CE *E. coli* and $\geq$10 CE *Enterococcus* spp./100 ml sample volume, independently of the culture conditions used. Therefore, qPCR is suitable to detect higher contamination levels rapidly. However, a single CE *E. coli* or *Enterococcus* spp. was not reliably detected. Hence, qPCR cannot be recommended as an adequate alternative for drinking water analysis. Instead, it can be used only as a monitoring tool (Whitman *et al.* 2010; Lam *et al.* 2014; Anonymous 2015; Mendes Silva & Domingues 2015). Nevertheless, qPCR could detect single viable *E. coli* or *Enterococcus* spp., after the application of enrichment cultures (Sen *et al.* 2011; Mendes Silva & Domingues 2015).

ACKNOWLEDGEMENTS

This research was funded by the Swiss Commission for Technology and Innovation (CTI, project: PFLS-LS 9251.1). We thank A. U. Kasper and J. Schmid for their support during the assay development and A. Peter for access to different water sampling sites in the canton of Zurich and for providing drinking water samples before UV disinfection. We further thank C. Wohler, P. Andrae, A. Gerber and V. Lachmann for excellent technical assistance.

REFERENCES

Ahmed, W., Richardson, K., Sidhu, J. P. S. & Toze, S. 2012 *Escherichia coli* and *Enterococcus* spp. in rainwater tank samples: comparison of culture-based methods and 23S rRNA gene quantitative PCR assays. *Environmental Science & Technology* **46**, 11370–11376.

Anonymous 1998 Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. L 330/32. *Official Journal of the European Communities*. http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:31998L0083&from=RO.

Anonymous 2005 Hygieneverordnung des EDI (Eidgenössisches Departement des Innern), HyV 817.024.1 vom 23. November 2005 (Stand am 01. Januar 2014).

Anonymous 2015 Method 1611.1: Enterococci in Water by TaqMan Quantitative Polymerase Chain Reaction (qPCR). US Environmental Protection Agency, April 2015.

Bergeron, P., Oujati, H., Catalán Cuena, V., Huguet Mestre, J. M. & Courtois, S. 2011 Rapid monitoring of *Escherichia coli* and *Enterococcus* spp. in bathing water using reverse transcription-quantitative PCR. *International Journal of Hygiene and Environmental Health* **214**, 478–484.

Berney, M., Vital, M., Hülshoff, I., Weilenmann, H. U., Egli, T. & Hammes, F. 2008 Rapid, cultivation-independent assessment of microbial viability in drinking water. *Water Research* **42**, 4010–4018.

Botes, M., de Kwaadsteniet, M. & Cloete, T. E. 2013 Application of quantitative PCR for the detection of microorganisms in water. *Analytical and Bioanalytical Chemistry* **405**, 91–108.

Bustin, S. A. & Nolan, T. 2004 Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *Journal of Biomolecular Techniques (JBT)* **15**, 155–166.

Converse, R. R., Griffith, J. F., Noble, R. T., Haugland, R. A., Schiff, K. C. & Weisberg, S. B. 2012 Correlation between quantitative PCR and culture-based methods for measuring *Enterococcus* spp. over various temporal scales at three California marine beaches. *Applied and Environmental Microbiology* **78**, 1237–1242.

Edberg, S. C., Rice, E. W., Karlin, R. J. & Allen, M. J. 2000 *Escherichia coli*: the best biological drinking water indicator for public health protection. *Journal of Applied Microbiology* **88**, 106–116.

Fittipaldi, M., Nocker, A. & Codony, F. 2012 Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. *Journal of Microbiological Methods* **91**, 276–289.

Frahm, E. & Obst, U. 2003 Application of the fluorogenic probe technique (TaqMan PCR) to the detection of *Enterococcus* spp. and *Escherichia coli* in water samples. *Journal of Microbiological Methods* **52**, 123–131.

Harwood, V. J., Delahoya, N. C., Ulrich, R. M., Kramer, M. F., Whitlock, J. E., Garey, J. R. & Lim, D. V. 2004 Molecular confirmation of *Enterococcus faecalis* and *E. faecium* from clinical, faecal and environmental sources. *Letters in Applied Microbiology* **38**, 476–482.

Hrudey, S. E., Payment, P., Huck, P. M., Gillham, R. W. & Hrudey, E. J. 2003 A fatal waterborne disease epidemic in Walkerton, Ontario: comparison with other waterborne outbreaks in the developed world. *Water Science and Technology* **47**, 7–14.

Keer, J. T. & Birch, L. 2003 Molecular methods for the assessment of bacterial viability. *Journal of Microbiological Methods* **53**, 175–183.

Lam, J. T., Lui, E., Chau, S., Kueh, C. S. W., Yung, Y. K. & Yam, W. C. 2014 Evaluation of real-time PCR for quantitative detection of *Escherichia coli* in beach water. *Journal of Water and Health* **12**, 51–56.

Ludwig, W. & Schleifer, K. 2000 How quantitative is quantitative PCR with respect to cell counts? *Systematic and Applied Microbiology* **23**, 556–562.

Maurer, A. M. & Stürchler, D. 2000 A waterborne outbreak of small round structured virus, *Campylobacter* and *Shigella* co-infections in La Neuveville, Switzerland, 1998. *Epidemiology and Infection* **125**, 325–332.

Mendes Silva, D. & Domingues, L. 2015 On the track for an efficient detection of *Escherichia coli* in water: a review on PCR-based methods. *Ecotoxicology and Environmental Safety* **113**, 400–411.

Nocker, A. & Camper, A. K. 2009 Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. *FEMS Microbiology Letters* **291**, 137–142.

Nocker, A., Cheung, C. Y. & Camper, A. K. 2006 Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *Journal of Microbiological Methods* **67**, 310–320.

Nocker, A., Sossa, K. E. & Camper, A. K. 2007 Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. *Journal of Microbiological Methods* **70**, 252–260.

Nogva, H. K., Dromtorp, S. M., Nissen, H. & Rudi, K. 2003 Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5'-nuclease PCR. *BioTechniques* **34**, 804–813.

Oliver, J. D. 2010 Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiology Reviews* **34**, 415–425.

Prüss-Üstün, A., Bos, R., Gore, F. & Bartram, J. 2008 *Safer Water, Better Health: Costs, Benefits and Sustainability of Interventions to Protect and Promote Health*. World Health Organization, Geneva, p. 7.

Ryu, H., Henson, M., Elk, M., Toledo-Hernandez, C., Griffith, J., Blackwood, D., Noble, R., Gourmelon, M., Glassmeyer, S. & Santo Domingo, J. W. 2013 Development of quantitative PCR assays targeting the 16S rRNA genes of *Enterococcus* spp. and their application to the identification of *Enterococcus* species in environmental samples. *Applied and Environmental Microbiology* **79**, 196–204.

Schaffner, M., Studer, P. & Ramseier, C. 2013 Beurteilung der Badegewässer-Empfehlungen zur Untersuchung und Beurteilung der Badewasserqualität von See- und Flussbädern. *Umwelt-Vollzug* **1310**, 1–42.

Sen, K., Sinclair, J. L., Boczek, L. & Rice, E. W. 2011 Development of a sensitive detection method for stressed *E. coli* O157: H7 in source and finished drinking water by culture-qPCR. *Environmental Science & Technology* **45**, 2250–2256.

Smith, C. J. & Osborn, A. M. 2009 Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiology Ecology* **67**, 6–20.

Varma, M., Field, R., Stinson, M., Rukovets, B., Wymer, L. & Haugland, R. 2009 Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater. *Water Research* **43**, 4790–4801.

Whitman, R. L., Ge, Z., Nevers, M. B., Boehm, A. B., Chern, E. C., Haugland, R. A., Lukasik, A. M., Molina, M., Przybyla-Kelly, K. & Shively, D. A. 2010 Relationship and variation of qPCR and culturable enterococci estimates in ambient surface waters are predictable. *Environmental Science & Technology* **44**, 5049–5054.

WHO 2011 *Guidelines for Drinking-Water Quality*. 4th edn. World Health Organization, Geneva.

Wohler, C., Krapf-Kovalj, T., Gantenbein-Demarchi, C. & Kuhn, R. 2012 WO 2012/001111 A2. *Method for determining organisms in water.* 05.01.2012.

First received 22 December 2015; accepted in revised form 29 March 2016. Available online 3 June 2016