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Evaluation of quantitative PCR combined with PMA treatment for molecular assessment of microbial water quality

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ABSTRACT

Microbial water quality assessment currently relies on cultivation-based methods. Nucleic acid-based techniques such as quantitative PCR (qPCR) enable more rapid and specific detection of target organisms and propidium monoazide (PMA) treatment facilitates the exclusion of false positive results caused by DNA from dead cells.

Established molecular assays (qPCR and PMA-qPCR) for legally defined microbial quality parameters (*Escherichia coli*, *Enterococcus* spp. and *Pseudomonas aeruginosa*) and indicator organism group of coliforms (implemented on the molecular detection of *Enterobacteriaceae*) were comparatively evaluated to conventional microbiological methods. The evaluation of an extended set of drinking and process water samples showed that PMA-qPCR for *E. coli*, *Enterococcus* spp. and *P. aeruginosa* resulted in higher specificity because substantial or complete reduction of false positive signals in comparison to qPCR were obtained. Complete compliance to reference method was achieved for *E. coli* PMA-qPCR and 100% specificity for *Enterococcus* spp. and *P. aeruginosa* in the evaluation of process water samples. A major challenge remained in sensitivity of the assays, exhibited through false negative results (7–23%), which is presumably due to insufficient sample preparation (i.e. concentration of bacteria and DNA extraction), rather than the qPCR limit of detection. For the detection of the indicator group of coliforms, the evaluation study revealed that the utilization of alternative molecular assays based on the taxonomic group of *Enterobacteriaceae* was not adequate.

Given the careful optimization of the sensitivity, the highly specific PMA-qPCR could be a valuable tool for rapid detection of hygienic parameters such as *E. coli*, *Enterococcus* spp. and *P. aeruginosa*.

Abbreviations: propidium monoazide (PMA), quantitative PCR (qPCR); limit of detection (LOD), quantification cycle (Cq).

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1. Introduction

In scope of routine quality analysis of water, microbiological parameters (*Escherichia coli*, coliforms, *Enterococcus* spp., *Pseudomonas aeruginosa*) are usually monitored by cultivation-based techniques on selective agar plates followed by biochemical confirmation tests. In general, those culture-based techniques are time consuming and laborious (Agudelo et al., 2010) in their execution i.e. several cultivation steps are necessary, which may require up to seven days e.g. for the confirmation of *P. aeruginosa*.

In recent years the potential of molecular DNA-based assays was recognized, enabling more rapid, specific and high-throughput detection of target organisms from a variety of matrices (Aw and Rose, 2012). Developed qPCR techniques for the detection of pathogens in water have been included in some governmental guidelines in the U.S. (Varma et al., 2009). Furthermore, numerous qPCR-based methods were proposed for microbial risk assessment in water (Layton et al., 2006; Revetta et al., 2010; Lamendella et al., 2007; Sivaganensan et al., 2012). However, to our best knowledge this approach has not yet been considered for the detection of the whole set of microbial parameters defined for water quality assessment.

As standard microbiological methods are based on viable cell detection, some adaptions of qPCR are of concern, because DNA-based methods have the innate inability to discriminate between DNA from living and dead bacterial cells. A combination of qPCR with propidium monoazide (PMA) treatment was previously investigated in several studies for specific monitoring of viable target bacteria (Nocker et al., 2007; Yáñez et al., 2011; Yokomachi and Yaguchi, 2012; Van Frankenhuyzen et al., 2013). PMA is a DNA intercalating molecule with the capacity to diffuse into dead or membrane compromised cells, thereby irreversibly modifying DNA by forming stable covalent nitrogen–carbon bonds upon photo-activation. Consequently, this modification inhibits PCR amplification of DNA from dead cells, allowing selective PCR amplification of unmodified DNA from viable cells (https://ca.vwr.com/store/catalog/product.jsp?product_id=8286393; Nocker and Camper, 2009). Successful application of PMA-qPCR for detection of *E. coli* and *P. aeruginosa* in complex water-related microbial matrices was shown previously in our studies, achieving substantial reduction (~3 logs) or complete suppression of amplification arising from DNA of dead cells (Gensberger et al., 2013).

Therefore this study focuses on the investigation of the application of molecular assays (qPCR and PMA-qPCR) to rapidly assess microbial water quality. qPCR-based assays were established and optimized for microbial parameters defined according to the Austrian Drinking Water Directive (DWD, 2001), i.e. *E. coli*, coliforms, *Enterococcus* spp., *P. aeruginosa*. Performance parameters (specificity and sensitivity) were comparatively determined to the respective standard microbiological method using a variety of drinking water and process water samples.

2. Material & methods

2.1. Water sample collection

Water samples were collected from multiple sources in urban and rural areas in Lower Austria, Vienna and Burgenland, Austria. In total 100 drinking water samples were collected, comprising of 65 well water samples, 16 spring water samples and 19 samples from public water supply. Further, process water application was tested with 30 process water samples collected from 16 cooling towers, 6 samples from a drinking water treatment plant and 8 samples from a constructed wetland. At all sites a total volume of 3 L was sampled according to DIN EN ISO 19458:2006 in sterile polypropylene plastic bottles (VWR, Austria). Samples were transported (refrigerated) to laboratory for analysis and stored at 4 °C until further processing (max. 18 h).

2.2. Standard water quality assessment

For water quality assessment as defined in the Austrian DWD (2001), reference methods were used such as the standard cultivation-based techniques defined in EN 12780:2002 and ISO 6222:1999 for the detection of *P. aeruginosa* and determination of heterotrophic plate counts (at 22 °C and 37 °C), respectively. For the detection of *Enterococcus* spp. and coliforms/*E. coli* alternatively approved chromogenic/fluorogenic tests (Enterolert®-DW and Colilert®-18; IDEXX, Austria) were used.

2.3. Sample preparation and PMA treatment for qPCR

For each molecular assay (qPCR and PMA-qPCR), 1 L water aliquot was filtered through a 0.45 μm nitrocellulose filter membrane (Millipore, Germany) and the bacteria were washed off with a 0.01% water-Tween20 solution. Bacterial cell suspension from the membrane filter of the first 1 L aliquot, intended for analysis with conventional qPCR, was pelleted by centrifugation at 10 000 × *g* for 5 min and subjected directly to DNA extraction. Bacterial cell suspension from the membrane filter of the second aliquot, intended for pre-treatment with PMA, was directly mixed with 10 μM PMA dye (Biotium Inc., USA). Firstly, samples were incubated for 5 min in dark, and then subsequently placed on ice and horizontally exposed to 500 W halogen light (distance 20 cm) for 3 min. After photo-activation, cells were pelleted at 10 000 × *g* for 5 min and DNA was extracted. For genomic DNA extraction the WaterMaster™ DNA Purification Kit (Epicentre, U.S.) was used, containing a specific inhibitor removal technology. Briefly, bacterial pellets from sample preparations were lysed by enzymatic treatment with lysozyme (45 mg/ml) and proteinase K (50 mg/ml). RNA was degraded by adding RNAse (5 mg/ml). DNA was precipitated with isopropanol followed by purification through a spin column (incl. inhibitor-removal step)and finally DNA was eluted in 60 μl sterile water.

2.4. Primers and probes for qPCR

For all microbiological parameters defined in the Austrian DWD (2001), PCR primers were selected from the published literature and extensively tested (with special focus on primer/probe specificity and limit of detection of the assays).

The complete list of tested primers can be found in the list S1, Supplementary Material. The list of selected and used primers and probes based on TaqMan® chemistry is given in Table 1, exclusively *Enterobacteriaceae* (coliform) detection was facilitated by intercalating dye technology (EvaGreen®, Estonia). As analogue to the bacterial enumeration by HPC assay, the quantification of total bacteria in the sample was attempted by targeting the phylogenetic bacterial marker of 16S rRNA gene, but no clear relation by comparing these methods could be ascertained (see Fig. S1, Supplementary Material). Nevertheless for inhibition testing (see Section 2.7), the quantification of total bacteria was utilized.

All primers and probes were tested *in silico* by retrieving sequence of target genes from public database (NCBI nucleotide database; http://www.ncbi.nlm.nih.gov/nuccore) and constructing primer/probe alignments using Bioedit program (version 7.2.1). In addition each primer and probe and potential amplicon sequence was checked by nucleotide BLAST analysis against the database hits with restriction of the target organism. For *in vitro* specificity a set of 26 bacterial target and non-target species with known association to water (Table 2) were used. They were accessed from the own strain collection containing environmental isolates or were purchased from DSMZ (www.dsmz.de). The identity of bacteria was re-confirmed by 16S rRNA gene sequencing with universal 16S rRNA primers 8f (5′-AGAGTTTGATCCTGGCTGAG-3′) and 518r (5′-ATTACCGCGGCTGCTGG-3′) and identified by BLAST.

For the *in vitro* specificity tests the genomic DNA from overnight cultures grown in 10% TSB (Merck, Austria) at 37 °C was isolated with the GenElute™ Bacterial Genomic DNA kit (Sigma Aldrich, Germany) based on enzymatic (lysozyme and proteinase K) treatment. DNA concentration was measured with Nanodrop1000 (Fisher Scientific, Austria) and adjusted to 25 ng μl^{-1}. Two µl (50 ng) were used as a template in the PCR reaction. Initially primers were tested in conventional PCR (using recombinant *Taq* DNA polymerase, Invitrogen, U.S. and following the conditions given in the original publication) and specificity was assessed via gel electrophoresis. Promising primers were further investigated in the qPCR. All qPCR experiments were performed in triplicates and included a non-template control (NTC).

2.5. Standard preparation for qPCR

Overnight cultures of *E. coli* DSM 30083, *P. aeruginosa* DSM 50071 and *Enterococcus faecalis* DSM 20478 were grown in liquid Luria Bertani medium (Sigma–Aldrich, Germany) and in Brain Heart Infusion broth (Sigma–Aldrich, Germany), respectively, all at 37 °C.

Enterobacteriaceae standard was prepared from the mixture of several species belonging to this family, i.e. *E. coli* DSM 30083, *Salmonella* spp. AIT-AM13, *Citrobacter* sp. DSM 30041, *Raoultella terrigena* DSM 2687, *Yersinia enterocolitica* DSM 11502, *Enterobacter asburiae* AIT-AM 9, *Shigella fexneri* DSM 4782 and

Table 1 – Oligonucleotide primers and probes used for qPCR analysis. Same primers and probes were used for PMA-qPCR.

Target name	Gen	Primer and probe name	Sequence [5′–3′]	Amplicon size (bp)	Reference
Intercalating dye – EvaGreen®					
Enterobacteriaceae[a]	23S rRNA	En-lsu-3-F	TGCCGTAACTTCGGGAGAAGGCA	428	Matsuda et al., 2009
		En-lsu-3-R	TCAAGGACCAGTGTTCAGTGTC		
TaqMan® system					
Total bacteria[b]	16S rRNA	331-F	TCCTACGGGAGGCAGCAGT	466	Nadkarni et al., 2002
		797-R	GGACTACCAGGGTATCTAATCCTGTT		
		probe	CGTATTACCGCGGCTGCTGGCAC		
E. coli & *Shigella* spp.	*uidA*	ECN1254-F	GCAAGGTGCACGGGAATATT	75	Takahashi et al., 2009
		ECN1328-R	CAGGTGATCGGACGCGT		
		probe	CGCCACTGGCGGAAGCAACG		
Enterococcus spp.	23S rRNA	ECST748-F	AGAAATTCCAAACGAACTTG	92	Haugland et al., 2005
		ENC854-R	CAGTGCTCTACCTCCATCATT		
		probe	T GGTTCTCTCCGAAATAGCTTTAGGGCTA		
Pseudomonas aeruginosa	*regA*	Paer-F	CTGCTGGTGGCACAGGA	64	Lee et al., 2008
		Paer-R	GTTGGTGCAGTTCCTCATTG		modified[c]
		probe	CCAGATGCTTTGCCTCAAGGTCGA		

[a] *Enterobacteriaceae* as alternative assay for coliform test.
[b] Quantification of total bacteria used for inhibition test.
[c] Modifications of primers and probes are shown with underscores.

Table 2 – List of bacteria that were used as reference strains for the primer specificity test.

Staphylococcus aureus SSM CI-1	*Comamonas acidovorans* AIT-AM 7
Escherichia coli DSM 30083	*Arthrobacter* spp. AIT-AM 3
Enterococcus faecalis DSM 20478	*Enterobacter asburiae* AIT-AM 9
Pseudomonas aeruginosa DSM 50071	*Bifidobacterium longum* AIT-AM 5
Clostridium perfringens DSM 756	*Bacteroides fragilis* AIT-AM 4
Salmonella spp. AIT-AM13	*Shigella flexneri* DSM 4782
Legionella pneumohila DSM 7513	*Mycobacterium tuberculosis* H37Rv
Citrobacter spp. DSM 30041	*Campylobacter jejuni* DSM 4688
Raoultella terrigena DSM 2687	*Streptococcus agalactiae* AIT-AM 6
Yersinia enterocolitica DSM 11502	*Helicobacter pylori* SSM 4138
Alcaligenes spp. AIT-AM 2	*Citrobacter freundii* CCM 4475
Acinetobacter calcoaceticus AIT-AM 1	*Klebsiella oxytoca* DSM 5175
Spingomonas paucimobilis AIT-AM 15	*Yersinia enterocolitica* DSM 4780

Citrobacter freundii CCM 4475. All cultures were grown over night in liquid plate count media (Sigma–Aldrich, Germany) at 37 °C. The standard for total bacteria (analysed by 16S rRNA gene analysis) was prepared from a well water sample that was filtered through a 0.45 μm filter (Millipore, Germany) and subsequently incubated on yeast extract agar at 37 °C (Sigma–Aldrich, Germany), according to the heterotrophic plate count method (ISO 6222:1999), and grown bacteria were washed off from filter for DNA extraction.

For preparation of standards, the genomic DNA was extracted with the GenElute™ Bacterial Genomic DNA kit (Sigma Aldrich, Germany). The concentration in standard stock solution was verified by determining the colony forming units (CFU/ml) by plating from used cultures. The concentration, given in cell equivalents (CE), was calculated considering the DNA elution volume. The corresponding standards were used in qPCR as serially diluted $\log_{10}$ transformed CE.

2.6. qPCR amplification

Hot FirePol EvaGreen® mix (Solis BioDyne, Estonia) and SsoFast™ Probes Supermix (Bio-Rad, Austria) were used for qPCR assays. Reaction volumes of 20 μl contained either 1× Hot FirePol EvaGreen® mix (see Table 1 for *Enterobacteriaceae*) or 1× SsoFast™ Probes Supermix, 0.5 μM primers and 0.25 μM probe and 5 μl DNA template from water sample. EvaGreen® system cycling conditions started with initial denaturation for 15 min at 95 °C, followed by 40 cycles with 30 s at 95 °C, 40 s 52 °C, 60 s at 72 °C and final 60 s at 86 °C. Melting curve analysis was performed after each PCR. For this, samples were heated at 95 °C for 60 s, cooled to 55 °C for 60 s and subsequently the temperature ramped from 55 °C to 95 °C for 60 s in 0.5 °C increments per cycle. Fluorescence was measured at the end of each cycle. The cycling parameters for TaqMan® assays were as follows: initial denaturation for 2 min at 95 °C, followed by 40 cycles with 5 s at 95 °C and 30 s at 62 °C (*E. coli*, *P. aeruginosa*, total bacteria) or 20 s at 60 °C (*Enterococcus* spp.).

The threshold baseline, slopes and efficiencies were automatically calculated by the Bio-Rad CFX Manager version 3.0 software (Biorad, Austria) by running a corresponding bacterial standard in a range of 10^6 to 10^0 CE.

2.7. Amplification inhibition tests

Inhibition of qPCR amplification reaction arising from the sample matrix can lead to false negative results (Staley et al., 2012), therefore potential inhibition of DNA from water samples was assessed. For this, an inhibition test based on dilution of DNA sample was selected. Briefly, undiluted and 1:10 diluted DNA samples were quantitatively analysed for the amount of total bacteria targeting 16S rRNA genes in qPCR. Higher quantitative values for diluted samples (1:10) in comparison to undiluted one indicated an inhibition, similar or lower quantities revealed no interference in amplification reaction. Samples showing an inhibition were tested with further dilutions (1:20 and 1:50) until an inhibition-free dilution was found that could be used for subsequent analysis.

2.8. Data analyses

The limit of detection (LOD), accordingly the sensitivity of qPCR, was verified from the 10-fold serial dilution of each prepared standard for all target organisms. The measured standard curves ranged from 10^6–10^0 CE and were examined in triplicates. NTC served as negative control, of possible contamination of PCR reagents in qPCR amplification runs. Amplification efficiencies were automatically calculated with the CFX software 3.0 (Biorad, Austria) with the formula $E = 10^{-1/s} - 1$, where s is the slope of the standard curves (Garrido et al., 2013). The quantification cycle (Cq) of the last detectable standard determined the LOD of the qPCR analysis in the case where NTC were not detectable. In the case where NTC showed signal in amplification, a cut-off principle was applied. Therefore LOD of these qPCRs were calculated according to the formula Cq (LOD) = Cq (NTC) – 3 (Caraguel et al., 2011). The samples with Cq values higher than Cq (LOD) were classified as non-determined and Cq values lower were classified as positive.

The overall detection limit of the molecular assays (including sample preparation and qPCR analysis) was estimated using pure cultures in logarithmic growth phase. 10-fold dilutions were prepared and 1 ml of the concentration range of 10^5–10^1 CFU was spiked in 1 L of drinking water. The corresponding bacterial counts were determined by plating and overall LOD verified from quantified CE with (PMA)-qPCR. qPCR analysis was performed in triplicates and extraction blanks were also included.

Assay repeatability was determined by analyzing the standard dilution series (10^6–10^0 CE) with five replicates per dilution. Experiments were repeated on three different days by the same operator and on the same CFX96™ cycler (Biorad, Austria).

The analytical performance characteristics of (PMA)-qPCR was evaluated through the specificity and sensitivity of the test. Therefore firstly the rates of true positives (TP) and true negatives (TN) and moreover the rates of false positives (FP) and negatives (FN) were determined. The results of standard water quality assessment were taken as a reference and considered as "true". Diagnostic sensitivity is the proportion of true positives (TP) and correct positives scored with the validated method, according to the formula: $Sensitivity = TP/(TP + FN)$. Specificity is the proportion of true negatives and

false positives samples assigned by the test, according to the formula: *Specificity* = *TN*/(*FP* + *TN*). According to the performance parameters the statistical difference of qPCR and PMA-qPCR was tested by Student's *t*-test. Further the accuracy of a method was described by the evaluation of calculated positive and negative predictive values (PPV and NPV). PPV indicates the probability that a positive test results correctly identifies the presence of a contamination (*PPV* = *TP*/(*TP* + *FP*)) while NPV indicates the probability that a negative result correctly identifies the absence of a contamination (*NPV* = *TN*/(*FN* + *TN*)). (Caraguel et al., 2011; Alberg et al., 2004; Nutz et al., 2011.

3. Results

3.1. Specificity test of selected primers/probes for qPCR

The focus of primer/probe specificity testing was on exclusivity, i.e. ensuring that selected primers will not yield false positive signals with species known to be native in water samples. Therefore a set of 26 bacteria was selected for specificity testing (Table 2).

Primers selected for the detection of *E. coli*/*Shigella* spp., *P. aeruginosa* and *Enterococcus* spp. were highly specific and yielded positive amplification results only with targeted species. For the detection of coliforms, first functional marker gene *lacZ* (encoding for β-galactosidase) was tested (Bej et al., 1990, 1991a, b), but amplification efficiency of tested coliform bacteria was insufficient. Therefore qPCR assay was implemented through targeting the taxonomic group of *Enterobacteriaceae* (Matsuda et al., 2009). *Enterobacteriaceae* encompass members of coliform groups but also other genera including pathogens like *Salmonella* spp. and *Yersinia* species. Selected PCR primers targeted 23S rRNA genes, and positive signals were obtained for all tested *Enterobacteriaceae*.

3.2. Limit of detection of qPCR

For selected qPCRs, the LOD and accordingly sensitivity was determined by analyzing a 10-fold serial dilution of the corresponding standard. The standard curves of *Enterobacteriaceae* and *Enterococcus* spp. were linear and robust over 6 log units from 10^6–10^1 CE. The standard dilution 10^0 had to be excluded from the analysis based on the cut-off established from NTC. Detectable signals from negative controls (Cq = 34–39) from runs targeting universal bacterial phylogenetic markers (16S rRNA or 23S rRNA genes) could be explained by the fact that polymerase preparations inevitably contain contaminating microbial DNA (Corless et al., 2000; Spangler et al., 2009). The qPCRs for *E. coli* and *P. aeruginosa* allowed the detection of 10^6–10^0 standard CE, because negative controls were not detected and accordingly no cut-offs had to be set.

Based on the qPCR LOD (10^0–10^1 CE), overall LOD of the analysis was estimated to be in the range 12–120 bacterial cells (under assumption of 100% DNA extraction efficiency). In order to determine overall LOD of molecular assays water samples (1 L) were spiked with 10–10^5 bacterial cells and submitted to complete experimental procedure (filtration, (PMA treatment), DNA extraction, qPCR). The results are presented in Table 3. Overall detection limit was determined to be 10^2–10^3 bacterial cells for qPCR and PMA-qPCR respectively, indicating that sample preparation accounts for approximately 1log sensitivity loss.

In course of the assay utilization also repeatability was determined. For example qPCR targeting *Enterococcus* spp. achieved efficiencies of 98–103% with high correlation coefficient of 0.997. The standard curves from three independent days confirmed the robust repeatability of the assay with ΔCq between ten-fold standard dilutions of 3.27–3.35 and standard deviations within one standard point of 0.065–0.190. Other qPCRs showed similar results and efficiency, slope and correlation coefficient are illustrated in Table 4.

3.3. Evaluation of PMA-qPCR and qPCR for water quality assessment

3.3.1. Standard water quality assessment

Water quality assessment was conducted according to the Austrian DWD (2001) with the legal definition that microbial quality parameters (*E. coli*, coliforms, *Enterococcus* spp. and *P. aeruginosa*) in 100 ml analysed drinking water sample have to be absent. Furthermore the maximal enumerated values of HPC of 20 colonies/ml for 37 °C and 100 colonies/ml for 22 °C

Table 3 – Determination of the overall limit of detection of molecular assays (qPCR/PMA-qPCR), herein illustrated on the example of *E. coli* detection.

Spiked CFU in 1 L water sample[a]	Cell equivalents (CE) according to qPCR		Cell equivalents (CE) extrapolated for 1 L water sample[b]	
	qPCR	PMA-qPCR	qPCR	PMA-qPCR
5×10^5	1.51×10^4	7.35×10^3	1.81×10^5	8.82×10^4
5×10^4	2.06×10^3	1.58×10^3	2.47×10^4	1.90×10^4
5×10^3	1.70×10^2	1.40×10^2	2.04×10^3	1.68×10^3
5×10^2	2.52×10^1	n.d[c]	3.02×10^2	n.d
5×10^1	n.d	n.d	n.d	n.d
Negative control	n.d	n.d	n.d	n.d

[a] Determined from plating of bacterial culture used for spiking.
[b] DNA elution volume of 60 μl and 5 μl of DNA template was used in (PMA)-qPCR. Accordingly CE were extrapolated (x 12) to the total value for the analyzed water sample.
[c] n.d – not determined.

Table 4 – Summary of qPCR amplification efficiency, slope of the standard curve and correlation coefficient values for all qPCR assays evaluated in the study.

	Efficiency[a] %	Slope	R^2 (correlation coefficient)
E. coli	99.4–109.8	−2.992 to −3.337	0.989–0.995
Enterobacteriaceae	89.9–101.7	−3.275 to −3.589	0.979–1.000
Enterococcus spp.	98.0–103.0	−3.275 to −3.422	0.997–0.999
P. aeruginosa	94.8–110.1	−3.026 to −3.452	0.994–0.999

[a] Efficiency automatically calculated from Biorad-CFX manager version 3.0 software according to $E = 10^{-1/s} - 1$.

must not be exceeded. Results of the standard microbiological methods (Fig. S2, Supplementary Material) revealed that 20 out of 100 drinking water samples and 6 out of 30 process waters were acceptable according to the Austrian DWD (2001). The most frequent contaminations found in drinking water samples were coliforms (69/100) followed by the exceeding of the threshold values from HPC 37 °C. Less abundant were positive samples for *E. coli* (22/100) and *P. aeruginosa* (20/100). For process water numerous HPC 37 °C exceedings (12/30) and further contaminations with coliforms and *Enterococcus* spp. with 9 positive samples out of 30 were determined. Microbiological reference values for confirmation of *P. aeruginosa* were not obtained for 8 samples collected from cooling towers because selective media plates were overgrown by a fungal contamination.

3.3.2. *Inhibition tests*

Performance assessment was based on 96 samples only, because in 4 samples molecular analysis was not possible due to problems during DNA extraction (too low elution volume achieved). First inhibition was tested; 24 (25%) drinking water samples and 17 (57%) process water samples exhibited inhibition. The highest inhibition rate was seen for well waters (15 samples) and waters from cooling towers (12 samples). For these samples all (PMA)-qPCR analysis were performed with 1:10 dilution of original DNA. However, the detected inhibition could not be directly assigned to the occurrence of false negative results for these samples. Another problem that might have affected the overall sensitivity of the molecular analysis was due to solid organic material blocking filtration. This occurred in three samples, from which only 450–700 ml were filtrated. However, these samples did not exhibit false negative results.

3.3.3. *Performance assessment of qPCRs (without and with PMA treatment)*

Results from both molecular assays were compared to each other and further to reference values obtained from standard water quality assessment (considered to be a "true" result). Conventional qPCR (without PMA treatment) was included to reveal PMA-induced reduction of false positives. Per legal definition in the Austrian DWD (2001) the absence (0/100 ml water) of *E. coli*, coliforms, *Enterococcus* spp., and *P. aeruginosa* has to be assured. Accordingly, the results from qPCRs were only qualitatively specified, presented as positive detections (amplification signal Cq < LOD) and non-determined (amplification signal Cq > LOD or even no signal detected).

The performances of both qPCR and PMA-qPCR for assessing the quality of drinking and process water samples are summarized in Table 5 and were depicted as percent agreement to reference test, false negatives and false positives for target bacteria.

Table 5 – Comparison of qPCR and PMA-qPCR results to reference results of conventional microbiological analysis (considered to be true values). Percentage of agreement, false negatives and false positives are given for all tested parameters.

Percentage [%]	*Enterobacteriaceae*	*E. coli*	*Enterococcus* spp.	*P. aeruginosa*
Drinking water (96 samples)				
qPCR				
Agreement	70	79	79	81
False negative	13	12	12	14
False positive	17	9	9	5
PMA-qPCR				
Agreement	66	84	75	84
False negative	22	13	22	15
False positive	12	3	3	1
Process water (30 samples)				
qPCR				
Agreement	76	90	73	73
False negative	7	0	17	18
False positive	17	10	10	9
PMA-qPCR				
Agreement	67	100	80	91
False negative	23	0	20	9
False positive	10	0	0	0

The compliance of results was on average 70–81% for qPCR and 66–84% for PMA-qPCR in drinking water evaluation. Best performance in evaluation was identified for *E. coli* and *P. aeruginosa* PMA-qPCRs both having correlation rates of 84%. The treatment with PMA, induced reduction of false positives, e.g. for *E. coli* a decrease of 6% was determined and for *P. aeruginosa* 4%. Further also for *Enterococcus* spp. the number of false positives in qPCR could be reduced to 6% with PMA-qPCR. However, for this assay the agreement (75%) was somewhat lower when compared to reference results.

Low performance was assigned to *Enterobacteriaceae* in both assays (qPCR and PMA-qPCR) because high rates of both false positives (12–17%) and false negatives (13–22%) were determined. These assays strongly indicate that the detection of the phylogenetic assigned *Enterobacteriaceae* group is not well correlating and can hardly be compared to biochemically characterized coliform group identified through reference test based on the activity of the enzyme β-galactosidase. However, it was reported that the used enzymatic based reference test, Colilert®-18, also accounts for some bias because activity of the enzyme is regulated by environmental factors. Furthermore, some non-coliform bacterial species can interfere with the chromogenic reaction (Maheux et al., 2008, 2014).

In process water evaluation better performance of qPCRs were observed for all indicator bacteria (with only exception of *Enterobacteriaceae*). Actually PMA-qPCR for the detection of *E. coli* demonstrated 100% agreement to results from reference test. Furthermore, no false positives could be assigned in PMA-qPCR for *Enterococcus* spp. and *P. aeruginosa*. Therefore higher confirmation to reference test was achieved with 91% (*P. aeruginosa*) and 80% (*Enterococcus* spp.) agreement of PMA-qPCRs.

However, both molecular assays (qPCR and PMA-qPCR) yielded a continuous amount of false negative results, in which target organisms were not determined with molecular analysis but were positive with reference test (Table 2). This bias was seen for both drinking and process water evaluation study and is assumed to be due to insufficient sample preparation (i.e. filtration, DNA extraction), leading to the loss of cells, especially for initially low numbers of target bacteria.

3.3.4. *Diagnostic parameters for PMA-qPCR and qPCR*

Diagnostic values and the performance characteristics of qPCR and PMA-qPCR are illustrated in Table 6.

Both assays resulted in relatively high specificity values, with improvement over 15% by PMA-qPCR, with significances of $p < 0.01^{*}$ (drinking water) and $p < 0.001^{**}$ (process water) compared to qPCR without PMA treatment. Accordingly, also the PPV were higher for PMA-qPCR either in drinking water and at best in process water evaluation with an optimization from 50% to 100% with PMA-qPCR. This improvement was achieved for *E. coli*, *Enterococcus* spp. and *P. aeruginosa* (PPV of 100%), which is explained by no false positives and therefore higher compliance to reference methods. Best performance through diagnostic evaluation of the assays was attributed to *E. coli* PMA-qPCR with a 100% sensitivity and specificity for process water. The only exception was the performance of *Enterobacteriaceae* detection with both qPCR and PMA-qPCR with low specificity and sensitivity.

Nevertheless, problems were identified in the sensitivity for all assays; with lowest of 26.3% in drinking water. This was further reflected in NPV values, for both assays with almost similar values, e.g. 84.7% in qPCR and 84.4% PMA-qPCR for *P. aeruginosa* in drinking water. Both diagnostic parameters are an issue of false negative assignments in the evaluated test and as considered before, probably caused by inefficient sample preparation rather than the insufficient LOD of qPCR. Comparable values of qPCR and PMA-qPCR indicated that PMA

Table 6 – Diagnostic parameters for qPCR and PMA-qPCR. Rates of sensitivity, specificity, negative (NPV) and positive predictive values (PPV) are illustrated for drinking and process water.

Diagnostic value [%]	*Enterobacteriaceae*	*E. coli*	*Enterococcus* spp.	*P. aeruginosa*
Drinking water samples (total 96)				
qPCR				
Specificity	44.8	87.8	82.0	93.5
Sensitivity	82.1	50.0	76.1	31.6
NPV	52.0	85.5	78.9	84.7
PPV	77.5	55.0	79.6	54.6
PMA-qPCR				
Specificity	58.6	96.0	94.0	98.7
Sensitivity	68.7	45.5	54.4	26.3
NPV	44.7	85.5	69.1	84.4
PPV	79.3	76.9	89.3	83.3
Process water samples (total 30)				
qPCR				
Specificity	71.4	88.9	85.7	87.5
Sensitivity	88.9	100.0	44.4	33.3
NPV	88.2	100.0	78.3	77.8
PPV	61.5	50.0	57.1	50.0
PMA-qPCR				
Specificity	81.0	100.0	100.0	100.0
Sensitivity	33.3	100.0	33.3	66.7
NPV	70.8	100.0	77.8	88.9
PPV	50.0	100.0	100.0	100.0

treatment does only have a slightly negative effect on overall sensitivity of the method, presumably due to an additional sample preparation step.

4. Discussion

Cultivation-based techniques for determination of microbial quality parameters have been applied for a long time and are well established standardized procedures (Brettar and Höfle, 2008; Aw and Rose, 2012). However, demands to develop and implement more rapid and specific technologies, such as qPCR technology, were made in last decades. Nevertheless, it has to be considered that qPCR does not allow the differentiation between living and dead bacterial cells and thus has poor comparability to cultivation techniques. Some studies already combined qPCR with DNA intercalating dyes such as PMA for viable cell detection (Delgado-Viscogliosi et al., 2005; Slimani et al., 2012), but to our knowledge, this method is still not applied for entire microbial parameter set required in water quality assessment according to Drinking Water Regulative framework. Therefore this study focused on the establishment and evaluation of qPCR with PMA treatment for all specific microbiological parameters defined in Austrian DWD (2001) with diverse and representative water samples. The implementation of a PMA-treatment was included into established qPCR assays and proof of principle was shown for indicator parameters in an abundant water background microflora successfully excluding dead cells from analysis (Gensberger et al., 2013).

In evaluation of PMA-qPCR with drinking water and process water samples, best performances were identified for *P. aeruginosa* and *E. coli* detection, achieved through the substantial reduction of false positives and therefore leading to high specificity (100% process water). The specificity of these assays is also suggested in the utilization of specific marker genes for these single indicators. *E. coli* was detected targeting the sequence encoding the β-glucuronidase, *uidA* (Takahashi et al., 2009) and the pathogenic island of the *regA* (Lee et al., 2008) provided the determination of *P. aeruginosa*. Especially, in the case of *E. coli* direct comparability to Colilert®-18 test is facilitated, because both target β-glucuronidase. *E. coli* qPCR also includes the detection of *Shigella* spp., because of high homology in the *uidA* sequence between the two species (97–98%). This is not surprising given that the genus status of *Shigella* spp. is actually phenotypic (the ability to cause a specific type of diarrhoea) and that from evolutionary perspective *Shigella* strains should be classified as *E. coli* (Pupo et al., 2000; Zhang and Lin, 2012).

Contrarily to the high specificity, a low sensitivity, caused by continuous detection of similar values of false negative (12–22%) was obtained. However, this is not only attributed to PMA-qPCR, since qPCR exhibited almost same sensitivity values. Exceptions with slightly higher false negative scores with PMA-qPCR were identified for *Enterococcus* spp., which could be due to a moderate cytotoxic effect of PMA to this target organism (Yáñez et al., 2011). In general the sensitivity problem is presumed in the sample preparation procedure, as it was experimentally observed, rather than the LOD of qPCRs. Molecular detection of bacteria from water requires concentration of bacteria and then extraction of DNA. Commonly applied method for concentration of bacteria from water is the filtration, because of the easy handling and inexpensive equipment. However, after filtration the resuspension of bacteria from filters is necessary, which might result in a moderate recovery bias. All these methodical steps are not 100% efficient and consequently cause loss of target bacteria that are already present in low amounts in water samples (Brettar and Höfle, 2008; Agudelo et al., 2010). In fact, the qPCR LOD itself is rather low (1–10 targets per qPCR reaction) and would, under assumption of 100% efficient sample preparation, allow for overall sensitivity corresponding to 12–120 bacterial cells. However, experimentally determined sensitivity of the complete assay was shown to be in the range 10^2–10^3 cells indicating inherent problems of sample preparation. Insufficient sample preparation is generally of major concern in the application of molecular assays and numerous studies revealed the inherently variable and inefficient recovery of DNA from kits utilizing spin filter columns (Lemarchand et al., 2005; Lloyd et al., 2010; Haugland et al., 2012; Staley et al., 2012). Optimization to higher sensitivity is required, which could be assumed in improvement of DNA extraction protocol or assembling the filtration, PMA treatment and DNA extraction to one procedure in order to prevent loss of bacteria in separate steps (Slimani et al., 2012).

In general poor correlation (specificity and sensitivity) was observed for molecular detection of *Enterobacteriaceae* (for both qPCR and PMA-qPCR) compared to results of enzymatic (β-galactosidase) coliform reference test (Colilert®-18). This is most probably due to the differences in target spectrum between the taxonomically assigned *Enterobacteriaceae* and biochemical characterized group of coliforms. Coliforms are described as rod-shaped, non-spore forming, gram-negative, oxidase positive, bacteria that are able to grow on bile salts and further ferment lactose with gas and acid production. Actually the definitions of coliforms differ slightly from total coliforms to thermo-tolerant species (Rompré et al., 2002). In contrast, the *Enterobacteriaceae* family encompasses the detection of coliforms but also a range of non-coliform genera like *Salmonella* spp. or *Yersinia* species. For the coliforms the primer development is more difficult, because it is a diverse group containing many genera and primers must be specific to exclude some closely related non-coliforms (Rompré et al., 2002). However, as mentioned before the specificity of the tested *lacZ* was rather poor, not allowing the detection of some species belonging to the coliform group. New primer design or searching for other target gene regions may allow the improvement of the detection of coliform group.

5. Conclusions

- Inclusion of PMA treatment in qPCR analysis resulted in substantial or complete reduction of false positives
- Utilization of functional markers achieved higher specificity than targeting universal markers (i.e. 16S rRNA and 23S rRNA genes)
- High specificity was demonstrated for defined microbial parameters (*E. coli*, *Enterococcus* spp. and *P. aeruginosa*).

Utilization of molecular assays for indicator group of coliforms proved to be more challenging
- Best performances was achieved for *E. coli* PMA-qPCR in process water (100% specificity and sensitivity)
- Implementation of PMA-qPCR in water quality assessment will still need further optimization, validation of the assay (intra and inter-laboratory) and preparation of standardized protocols for legal harmonization
- PMA-qPCR can easily be extended to encompass other indicators or even pathogens

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.09.022.

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