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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.