



Notes & Tips

Propidium monoazide–quantitative polymerase chain reaction for viable *Escherichia coli* and *Pseudomonas aeruginosa* detection from abundant background microflora



Eva Theres Gensberger*, Angela Sessitsch, Tanja Kostić

AIT Austrian Institute of Technology, Bioresources Unit, A-3430 Tulln, Austria

ARTICLE INFO

Article history:

Received 30 March 2013
Received in revised form 28 May 2013
Accepted 31 May 2013
Available online 10 June 2013

Keywords:

Propidium monoazide
Quantitative PCR
P. aeruginosa
E. coli
Live/dead differentiation
Complex background microflora

ABSTRACT

Nucleic acid-based techniques represent a promising alternative to cultivation-based microbial water quality assessment methods. However, their application is hampered by their innate inability to differentiate between living and dead organisms. Propidium monoazide (PMA) treatment was proposed as an efficient approach for alleviating this limitation. In this study, we demonstrate the performance of PMA–quantitative polymerase chain reaction (qPCR) for the detection of indicator organisms (*Escherichia coli* and *Pseudomonas aeruginosa*) in a background of a highly abundant and complex microflora. Treatment with 10 μ M PMA resulted in the complete or significant reduction of the false positive signal arising from the amplification of DNA from dead cells.

© 2013 Elsevier Inc. All rights reserved.

Conventional water quality assessment parameters and corresponding detection methods are defined by the European Union in Council Directive 98/83/EC on the quality of water intended for human consumption [1]. Similar practices are also used in other countries (e.g., United States [2], Australia [3]). In general, mandatory methods are based on the cultivation of microorganisms and accordingly are very time-consuming. Some analyses may even require up to 7 days (e.g., the confirmation of *Pseudomonas aeruginosa* [4]). Another recently recognized disadvantage of cultivation-based methods is their inability to detect viable but nonculturable cells [5,6].

During the past few years, extensive discussions regarding the limitations of conventional and potential of novel nucleic acid-based methods have evolved [7–10]. Even though nucleic acid-based methods such as polymerase chain reaction (PCR)¹ exhibit a whole range of advantages such as speed, specificity, multiplexing, and automation potential, one major disadvantage that remains is the inability to differentiate between living and dead cells [11]. The inclusion of live/dead differentiation into nucleic acid-based assays is an absolute prerequisite for application of these methods for microbial water quality assessment.

DNA intercalating dyes, such as propidium monoazide (PMA), may be used for live/dead differentiation. Briefly, PMA is cell membrane impermeable and, therefore, can be used to selectively modify (i.e., bind to) free DNA and DNA from cells with compromised membrane integrity. By photo-activation, the PMA molecule forms a stable covalent nitrogen–carbon bond with a DNA molecule, resulting in irreversible DNA modification. This modification inhibits PCR amplification of DNA from dead cells, allowing selective PCR amplification of unmodified DNA from viable cells [12,13]. Performance of quantitative PCR (qPCR), including PMA treatment (PMA–qPCR), has been studied using pure cultures of single waterborne organisms [12,14–19]. However, a major challenge is a complex microbial community usually occurring in natural samples. For real-life applications, it is essential to demonstrate that reliable detection of few relevant organisms in a complex microbial background can be achieved. Therefore, our aim was to establish and test a PMA–qPCR assay that allows the detection of viable water quality indicators (*Escherichia coli* and *P. aeruginosa*) in a complex microbial background.

Overnight cultures of *E. coli* (DSM 30083) and *P. aeruginosa* (DSM 50071) were grown at 37 °C in liquid Luria–Bertani medium (Sigma–Aldrich, Germany) and Brain Heart Infusion broth (Sigma–Aldrich), respectively. A 10-fold dilution series was prepared for each organism, and cultivable cell numbers were estimated by plating on corresponding agar plates. Heat-killed cells were generated by heat inactivation at 75 °C for 10 min, and loss of viability

* Corresponding author. Fax: +43 050 550 3666.

E-mail address: eva.gensberger@ait.ac.at (E.T. Gensberger).

¹ Abbreviations used: PCR, polymerase chain reaction; PMA, propidium monoazide; PMA–qPCR, propidium monoazide–quantitative polymerase chain reaction; rRNA, ribosomal RNA.

was also confirmed by plate counting. For the detection and quantification of *E. coli* and *P. aeruginosa*, qPCR assays based on the *uidA* [20] and *regA* [21] genes were used. qPCR was performed using SsoFast Probes Supermix (Bio-Rad, Austria), 0.25 μ M probe and 0.5 μ M primers, and 2 μ l of total genomic DNA as a template. Cycling conditions 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.

The background microflora was prepared from a well water sample that was filtered through a 0.45- μ m filter (Millipore, Germany) and subsequently incubated on yeast extract agar (Sigma–Aldrich) according to the heterotrophic plate count method [22]. The diversity of obtained bacterial community was determined by 16S ribosomal RNA (rRNA) gene sequence analysis. Briefly, primers 8f (5'-AGAGTTTGATCCTGGCTGAG-3') and 1520r (5'-AAGGAGGTGATCCAGCCGCA-3') were used for the amplification of the 16S rRNA gene [23,24]. Amplicons were cloned using a StrataClone Cloning Kit (Agilent, Germany) and sent to Agowa (Germany) for sequencing with T3 (5'-AATTAACCCTCACTAAA GGG-3') and T7 (5'-TAATACGACTCACTATAGG-3') primers. The absence of *P. aeruginosa* and *E. coli* from the sample was tested by cultivation [4] or an enzymatic-based assay (Colilert 18, IDEXX Laboratories, Germany) and qPCR.

The efficiency of PMA (Biotium, USA) treatment was tested using artificial bacterial mixtures (Table 1). Each mixture consisted of a viable background microflora in excess (2–3 \log_{10} more than the target organism) and either viable, heat-killed, or both cultures of targeted organism(s). Two different PMA concentrations, 10 and 50 μ M, previously reported as optimal concentrations for live/dead differentiation [18,25] were tested. Briefly, samples were incubated with PMA in the dark for 5 min on ice, followed by photo-activation with a 500-W halogen light for 5 min on ice. Subsequently, DNA was isolated with a WaterMaster DNA Purification Kit (Epicentre, USA) according to the manufacturer's instructions

and eluted in 100 μ l of sterile water. Finally, 2 μ l was used as a template in qPCR assay.

To reflect the naturally occurring conditions as much as possible, a background microflora sample was prepared from well water. The community composition of the sample was analyzed by 16S rRNA sequence analysis, and the data revealed dominance of two bacterial phyla: Proteobacteria (89%) and Firmicutes (11%). The highest diversity was seen in the family of Enterobacteriaceae with *Citrobacter* spp. (53%) as the most abundant bacterial genus. Additional genera, including *Serratia* spp. (3%), *Klebsiella* spp. (3%), *Cedecea* spp. (2%), and *Hafnia* spp. (2%), were detected. In addition, *Bacillus* spp. (10%), *Aeromonas* spp. (3%), and *Vogesella* spp. (2%) were found. For some sequences (3%), unambiguous resolution down to the genus level was not achieved; however, these were identified as members of the Enterobacteriaceae, Moraxellaceae, and Bacillaceae families. These findings clearly demonstrate the complexity and relevance (presence of both Gram-positive and Gram-negative microorganisms) of the used background microflora.

The absence of *P. aeruginosa* and *E. coli* in the background microflora sample was confirmed by qPCR and through conventional assays.

In the preliminary study (data not shown), the optimal concentration of PMA for intended application was determined. For this purpose, a single indicator spike of *P. aeruginosa* (1.5×10^5 viable, heat-killed, or both cells) in the background microflora (10^8 viable cells) was used. Complete reduction of false positive signal ($\sim 3 \log_{10}$ units) obtained from the heat-killed cells was achieved. Better performance was observed with 10 μ M than with 50 μ M PMA concentration because the higher concentration showed stronger cytotoxic effects on viable cells, as reported previously [18]. Therefore, the following experiments were performed with 10 μ M PMA.

Table 1
Sample composition of artificial mixtures containing listed colony-forming unit amounts (as determined by plate count method) and PMA-induced signal/cell number reduction from conventional qPCR (without PMA) compared with PMA–qPCR (10 μ M) in high multiple indicator spike (A) and low multiple indicator spike (B)

Sample composition ^a		Viable		Mix		Dead	
<i>(A) High indicator spike: P. aeruginosa and E. coli in background microflora</i>							
Spike CFU	Viable cells (<i>P. aeruginosa</i>)	7.0E+05		7.0E+05		x	
	Heat-killed cells (<i>P. aeruginosa</i>)	x		7.0E+05		7.0E+05	
	Viable cells (<i>E. coli</i>)	8.0E+05		8.0E+05		x	
	Heat-killed cells (<i>E. coli</i>)	x		8.0E+05		8.0E+05	
	Background microflora	1.0E+08		1.0E+08		1.0E+08	
<i>P. aeruginosa</i> qPCR		Cq \pm SD	Cells	Cq \pm SD	Cells	Cq \pm SD	Cells
PMA (μ M)	0	24.74 \pm 0.017	3.9E+03	25.49 \pm 0.030	2.2E+03	30.37 \pm 0.116	5.5E+01
	10	27.20 \pm 0.055	6.1E+02	26.99 \pm 0.093	7.2E+02	<LOD ^b	<LOD ^b
<i>E. coli</i> qPCR							
PMA (μ M)	0	24.66 \pm 0.224	1.5E+04	25.45 \pm 0.057	8.6E+03	29.33 \pm 0.164	5.3E+02
	10	26.34 \pm 0.131	4.5E+03	25.93 \pm 0.178	6.1E+03	31.19 \pm 0.170	1.4E+02
<i>(B) Low indicator spike: P. aeruginosa and E. coli in background microflora</i>							
Spike CFU	Viable cells (<i>P. aeruginosa</i>)	5.4E+03		5.4E+03		x	
	Heat-killed cells (<i>P. aeruginosa</i>)	x		5.4E+03		5.4E+03	
	Viable cells (<i>E. coli</i>)	1.6E+03		1.6E+03		x	
	Heat-killed cells (<i>E. coli</i>)	x		1.6E+03		1.6E+03	
	Background microflora	5.0E+05		5.0E+05		5.0E+05	
<i>P. aeruginosa</i> qPCR		Cq \pm SD	Cells	Cq \pm SD	Cells	Cq \pm SD	Cells
PMA (μ M)	0	26.65 \pm 0.279	3.5E+02	26.03 \pm 0.131	5.4E+02	27.80 \pm 0.327	1.6E+02
	10	30.05 \pm 0.225	3.4E+01	30.40 \pm 0.104	2.7E+01	<LOD ^b	<LOD ^b
<i>E. coli</i> qPCR							
PMA (μ M)	0	28.90 \pm 0.265	1.2E+02	28.27 \pm 0.138	1.8E+02	30.04 \pm 0.263	6.6E+01
	10	30.80 \pm 0.131	3.4E+01	31.98 \pm 0.178	1.6E+01	<LOD ^b	<LOD ^b

Note. CFU, colony-forming units; Cq, quantification cycle; SD, standard deviation. SDs are calculated from three independent replicates.

^a Fifty times (50 \times) lower template amounts were used for qPCR analysis (under the assumption of 100% DNA extraction efficiency).

^b The limit of detection (LOD) of qPCR analysis (10 cells) is considered.

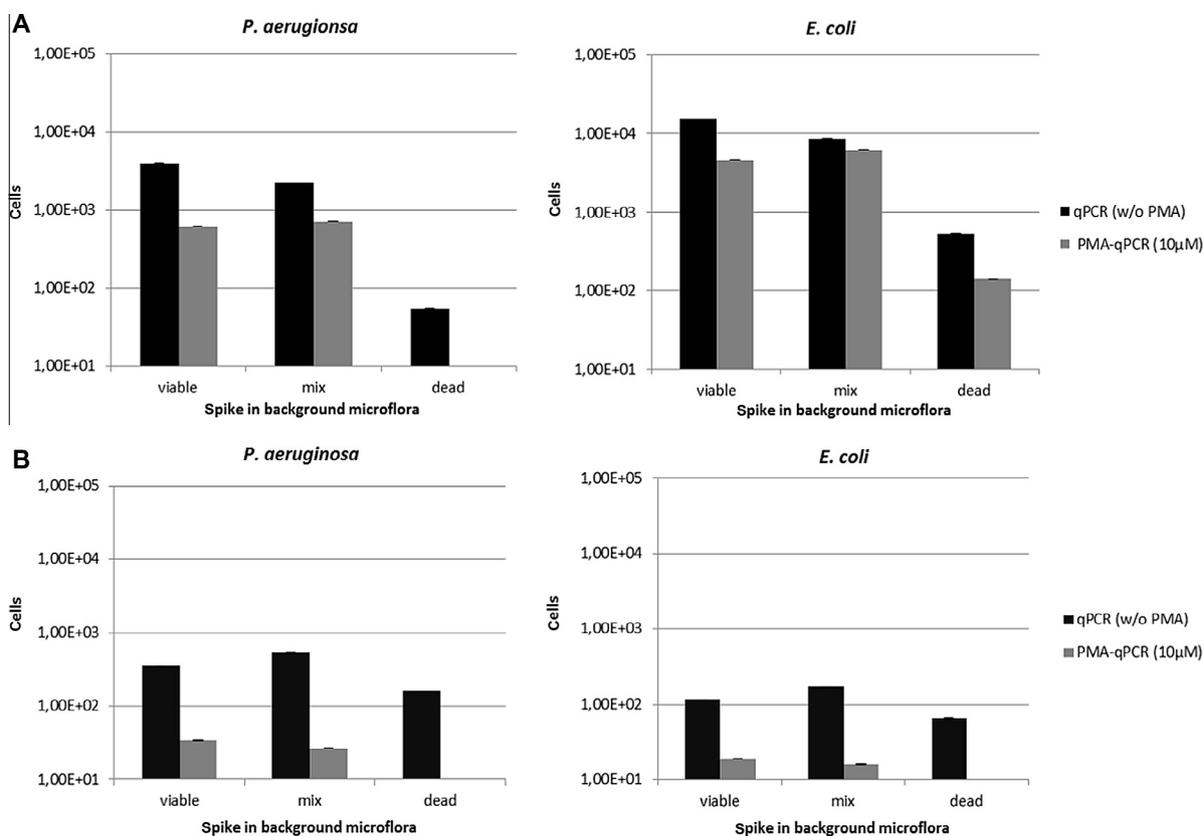


Fig. 1. Impact of PMA–qPCR (10 µM) compared with conventional qPCR (without PMA) in a high multiple indicator spike (A) and a low multiple indicator spike (B) of heat-killed and viable *P. aeruginosa* and *E. coli* in a viable background microflora. The background microflora sample was *P. aeruginosa* and *E. coli* negative (data not shown). Error bars represent standard deviations from three independent replicates. The limit of detection of qPCR analysis (10 cells) is considered.

In case of the high multiple indicator spike (Table 1A and Fig. 1A), PMA treatment resulted in complete suppression of false positive signals arising from heat-killed *P. aeruginosa* cells. For *E. coli*, only partial inhibition of qPCR signal from heat-killed cells could be achieved. There are two possible explanations for this. First, as shown previously, higher levels of dead cells in the mixture seem to have a negative effect on PMA performance [18,25,26]. In fact, the reported limit of PMA differentiation capacity lies approximately 10^4 to 10^5 dead cells and is close to the amounts used in this experiment. However, this spike was intentionally set to this elevated level in order to compensate for other potential biases such as insufficient sample preparation [27,28]. Another explanation could be previously observed species- and sequence-dependent differences in the efficiency of PMA binding and treatment efficiency [29].

To further demonstrate the usefulness of the proposed approach, low multiple indicator spike was prepared and analyzed. In this case, the concentration of background microflora was somewhat lower (5×10^5) and representative for naturally occurring raw waters [30]. Targeted bacteria were also spiked at significantly lower levels (1.6×10^3 and 5.4×10^3); consequently, also in this case, high abundance of background microflora was ensured (Table 1B). As expected, at this lower cell concentration, even better PMA performance was observed. Accordingly, complete signal reduction of false positive signals from heat-killed cells was achieved for both *P. aeruginosa* and *E. coli* (Table 1B and Fig. 1B). The observed negative effects were also somewhat greater; however, no false negative results were observed.

At this point, it must be emphasized that the water quality assessment does not require quantitative determination of the indicator organisms. Actually, regulatory norms define zero tolerance [1–3]. Therefore, it is only important to ensure the correct

detection of presence or absence. The presented data clearly demonstrate that conventional qPCR (without PMA) would always result in false positive detection of heat-killed bacteria. With implementation of PMA (10 µM), this could be alleviated in four of five cases. Only high spike of heat-killed *E. coli* would be diagnosed as falsely positive with the proposed method, and this sample is, as discussed above, not really representative for intended application. On the other hand, even in the case of low spike, no false negatives were observed.

In conclusion, the described PMA–qPCR approach resulted in the significant suppression of false positive signals arising from the amplification of DNA from the dead cells in conventional qPCR. It must be emphasized that these results were achieved in the presence of abundance (2–3 \log_{10} higher concentration than targeted species) and a complex background microflora. Therefore, we conclude that, given the careful optimization and validation, PMA–qPCR can be a valuable tool for monitoring microbial water quality parameters.

Acknowledgment

This study was partially funded by FEMtech project CHANGES (Federal Ministry for Transport, Innovation and Technology; Vienna University of Technology).

References

- [1] European Union, European Council Directive 98/83/EC of 3 November 1998 relating to the quality of water intended for human consumption, OJEC (Official Journal of the European Communities) L330/32–54.
- [2] Environmental Protection Agency, U.S. Water Directive. Available at: <http://water.epa.gov/scitech/methods/cwa/methods_index.cfm>.

- [3] National Health and Medical Research Council, Australian Drinking Water Guidelines Paper 6, National Water Quality Management Strategy, National Health and Medical Research Council, National Resource Management Ministerial Council, Commonwealth of Australia, Canberra, 2011.
- [4] European Committee for Standardization, EN 12780, Water Quality: Detection and Enumeration of *Pseudomonas aeruginosa* by Membrane Filtration, European Committee for Standardization, Brussels, Belgium, 2002.
- [5] L. Bjergbaek, P. Roslev, Formation of non-culturable *Escherichia coli* in drinking water, *J. Appl. Microbiol.* 99 (2005) 1090–1098.
- [6] J.D. Oliver, The viable but nonculturable state in bacteria, *J. Microbiol.* 43 (2005) 93–100.
- [7] O. Savitchcheva, S. Okabe, Alternative indicators of fecal pollution: relations with pathogens and conventional indicators, current methodologies for direct pathogen monitoring, and future application perspectives, *Water Res.* 40 (2006) 2463–2476.
- [8] L. Fiksdal, I. Tryland, Application of rapid enzyme assay techniques for monitoring of microbial water quality, *Curr. Opin. Biotechnol.* 19 (2008) 289–294.
- [9] R. Girones, M.A. Ferrús, J.L. Alonso, J. Rodriguez-Manzano, B. Calgua, A.A. Corrêa, A. Hundesa, A. Carratala, S. Bofill-Mas, Molecular detection of pathogens in water: the pros and cons of molecular techniques, *Water Res.* 44 (2010) 4325–4339.
- [10] G.A. Tiong, J.B. Rose, Detection of pathogens in water: from phylochips to qPCR to pyrosequencing, *Curr. Opin. Biotechnol.* 23 (2012) 422–430.
- [11] A. Nocker, C.Y. Cheung, A.K. Camper, Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells, *J. Microbiol. Methods* 67 (2006) 310–320.
- [12] A. Nocker, A.K. Camper, Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques, *FEMS Microbiol. Lett.* 291 (2009) 137–142.
- [13] VWR, PMA (propidium monoazide), Biotium. Available at: <http://ca.vwr.com/store/catalog/product.jsp?product_id=8286393>.
- [14] A. Nocker, K.E. Sossa, A.K. Camper, Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR, *J. Microbiol. Methods* 70 (2007) 252–260.
- [15] D.M. Cawthorn, R.C. Witthuhn, Selective PCR detection of viable *Enterobacter sakazakii* cells utilizing propidium monoazide or ethidium bromide monoazide, *J. Appl. Microbiol.* 105 (2008) 1178–1185.
- [16] B. Chang, T. Taguri, K. Sugiyama, J. Amemura-Maekawa, F. Kura, H. Watanabe, Comparison of ethidium monoazide and propidium monoazide for the selective detection of viable *Legionella* cells, *Jpn. J. Infect. Dis.* 63 (2010) 1119–1123.
- [17] B. Taskin, A.G. Gozen, M. Duran, Selective quantification of viable *Escherichia coli* bacteria in biosolids by quantitative PCR with propidium monoazide modification, *Appl. Environ. Microbiol.* 77 (2011) 4329–4335.
- [18] M.A. Yáñez, A. Nocker, E. Soria-Soria, R. Múrtula, L. Martínez, V. Catalán, Quantification of viable *Legionella pneumophila* cells using propidium monoazide combined with quantitative PCR, *J. Microbiol. Methods* 85 (2011) 124–130.
- [19] N. Yokomachi, J. Yaguchi, Enumeration of viable *Escherichia coli* by real-time PCR with propidium monoazide, *Water Sci. Technol.* 66 (2012) 2065–2073.
- [20] H. Takahashi, B. Kimura, Y. Tanaka, J. Shinozaki, T. Suda, T. Fujii, Real-time PCR and enrichment culture for sensitive detection and enumeration of *Escherichia coli*, *J. Microbiol. Methods* 79 (2009) 124–127.
- [21] D.Y. Lee, H. Lauder, H. Cruwys, P. Falletta, L.A. Beaudette, Development and application of an oligonucleotide microarray and real-time quantitative PCR for detection of wastewater bacterial pathogens, *Sci. Total Environ.* 398 (2008) 203–211.
- [22] International Organization for Standardization, ISO 6222, Water Quality: Enumeration of Culturable Micro-organisms—Colony Count by Inoculation in a Nutrient Agar Culture Medium, International Organization for Standardization, Geneva, Switzerland, 1999.
- [23] U. Edwards, T. Rogall, H. Blöcker, M. Emde, E.C. Böttger, Isolation and direct complete nucleotide determination of entire genes: characterization of a gene coding for 16S ribosomal RNA, *Nucleic Acids Res.* 17 (1989) 7843–7853.
- [24] A.A. Massol-Deya, D.A. Odelson, R.F. Hickey, J.M. Tiedje, Bacterial community fingerprinting of amplified 16S and 16–23S ribosomal gene sequences and restriction endonuclease analysis (ARDRA), in: *Molecular Microbial Ecology Manual* 3.3.2, Kluwer, Dordrecht, Netherlands, 1995, pp. 1–8.
- [25] S. Slimani, A. Robyns, S. Jarraud, M. Molmeret, E. Dusserrer, C. Mazure, J.P. Facon, G. Lina, J. Etienne, C. Ginevra, Evaluation of propidium monoazide (PMA) treatment directly on membrane filter for the enumeration of viable but noncultivable *Legionella* by qPCR, *J. Microbiol. Methods* 88 (2012) 319–321.
- [26] T. Løvdaal, M.B. Hovda, B. Björklom, S.G. Møller, Propidium monoazide combined with real-time quantitative PCR underestimates heat-killed *Listeria innocua*, *J. Microbiol. Methods* 85 (2011) 164–169.
- [27] K. Lemarchand, F. Berthiaume, C. Maynard, J. Harel, P. Payment, P. Bayardelle, L. Masson, R. Brousseau, Optimization of microbial DNA extraction and purification from raw wastewater samples for downstream pathogen detection by microarrays, *J. Microbiol. Methods* 63 (2005) 115–126.
- [28] K.G. Lloyd, B.J. Macgregor, A. Teske, Quantitative PCR methods for RNA and DNA in marine sediments: maximizing yield while overcoming inhibition, *FEMS Microbiol. Ecol.* 72 (2010) 143–151.
- [29] M. Fittipaldi, A. Nocker, F. Codony, Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification, *J. Microbiol. Methods* 91 (2012) 276–289.
- [30] F. Hammes, M. Berney, Y. Wang, M. Vital, O. Köster, T. Egli, Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes, *Water Res.* 42 (2008) 269–277.