Journal of Microbiological Methods 91 (2012) 276–289

Contents lists available at SciVerse ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Review

Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification ☆

Mariana Fittipaldi [a,*], Andreas Nocker [b], Francesc Codony [a,*]

[a] Laboratori de Microbiologia Sanitària i Mediambiental (MSM-Lab), Universitat Politècnica de Catalunya, Edifici GAIA, Rambla Sant Nebridi, 08222 Terrassa, Barcelona, Spain
[b] Cranfield Water Science Institute, Cranfield University, Cranfield, Bedfordshire, MK43 0AL, UK

ARTICLE INFO

Article history:
Received 11 June 2012
Received in revised form 16 August 2012
Accepted 16 August 2012
Available online 23 August 2012

Keywords:
Ethidium monoazide
Propidium monoazide
Viable cell detection
Viability PCR

ABSTRACT

The ideal scenario in most applications of microbial diagnostics is that only viable cells are detected. Bacteria were traditionally considered viable when they could be cultured, whereas today's viability concept tends to be alternatively based on the presence of some form of metabolic activity, a positive energy status, responsiveness, detection of RNA transcripts that tend to degrade rapidly after cell death, or of an intact membrane. The latter criterion, although conservative, was the focus of one of the most successful recent approaches to detect viable cells in combination with DNA amplification techniques. The technology is based on sample treatment with the photoactivatable, and cell membrane impermeant, nucleic acid intercalating dyes ethidium monoazide (EMA) or propidium monoazide (PMA) followed by light exposure prior to extraction of DNA and amplification. Light activation of DNA-bound dye molecules results in irreversible DNA modification and subsequent inhibition of its amplification. Sample pretreatment with viability dyes has so far been mainly used in combination with PCR (leading to the term viability PCR, v-PCR), and increasingly with isothermal amplification method. The principle is not limited to bacteria, but has also successfully been applied to fungi, protozoa and viruses. Despite the success of the method, some practical limitations have been identified, especially when applied to environmental samples. In part they can be minimized by choice of experimental parameters and conditions adequate for a particular sample. This review summarizes current knowledge and presents aspects which are important when designing experiments employing viability dyes.

Contents

1. Introduction . . . 277
2. Interaction of viability dyes with DNA and mechanism of amplification signal suppression . . . 278
   2.1. Comparison of photoreactive dyes with their non-reactive analogs . . . 278
   2.2. Mechanism of amplification signal suppression . . . 278
3. Specificity of viability dyes for live and dead cells . . . 278
   3.1. Exclusion of viability dyes from live cells . . . 278
      3.1.1. Fluorescence microscopy . . . 279
      3.1.2. qPCR signal reduction . . . 279
      3.1.3. Cytotoxicity . . . 279
   3.2. Exclusion of dead cell signals . . . 279
   3.3. Potential improvements to increase specificity . . . 280
      3.3.1. EMA . . . 280
      3.3.2. PMA . . . 280
   3.4. Summary of dye specificity characteristics . . . 280
4. Dye incubation conditions . . . 280
   4.1. Incubation time . . . 281
   4.2. Incubation temperature . . . 281

☆ Sponsor: Prof. Jack Trevors, jtrevors@uoguelph.ca.

* Corresponding authors at: Laboratori de Microbiologia Sanitària I Mediambiental, Universitat Politècnica de Catalunya, TR 14, Edifici GAIA, Rambla Sant Nebridi s/n 08222, Terrassa, Barcelona, Spain. Tel.: +34 937398566.
E-mail addresses: mariana@oo.upc.edu (M. Fittipaldi), francesc.codony@upc.edu (F. Codony).

0167-7012/$ – see front matter
http://dx.doi.org/10.1016/j.mimet.2012.08.007

5. Photoactivation . . . 281
5.1. Light source . . . 281
5.2. Light exposure time . . . 282
6. Target gene . . . 282
6.1. Length of the target gene . . . 282
6.2. Target gene sequence . . . 283
7. Species-specific differences in vPCR . . . 284
8. Matrix characteristics . . . 284
8.1. Presence of dead cells . . . 284
8.2. Salt concentration . . . 284
8.3. Turbidity . . . 285
8.4. pH . . . 285
9. Summary . . . 286
Disclosure statement . . . 287
References . . . 287

1. Introduction

Molecular methods targeting nucleic acids have revolutionized microbial detection. DNA-based methods, such as polymerase chain reaction (PCR), are rapid, versatile, sensitive, precise, and allow specific detection and/or quantification of microorganisms of interest in food, environmental, and clinical samples. Despite these advantages broad application is still hampered by some challenges. Apart from inhibition of amplification by substances naturally found in many environmental samples, the inability to differentiate between viable and nonviable cells and the resulting overestimation of microbial targets is considered a major disadvantage of PCR (Wang and Levin, 2006). Whereas the first limitation is greatly remedied by the incorporation of internal amplification controls, the second one was addressed by amplification of RNA instead of DNA. In contrast to the highly persistent DNA, RNA degrades more rapidly after cell death (Alifano et al., 1994). Detection of RNA and especially of the highly unstable mRNA thus tends to indicate the presence of live cells far better than the detection of DNA. In addition, messenger RNA (mRNA) is only produced by metabolically active cells, making mRNA suitable to specifically detect living microorganisms (Bleve et al., 2003; Morin et al., 2004). Nevertheless the same labile nature qualifying mRNA as a suitable target for detecting live cells at the same time makes working with it more challenging. Degradation can occur by inadequate sample processing and storage or as a result of sample contamination with RNA-degrading enzymes. Quantification of live cell numbers is further complicated by the fact that the expression levels of many mRNA species greatly depend on the physiological status of the cells, which is typically an unknown factor. It is likely that when slow-growing or dormant cells are present in a sample the RNA content of such cells is beneath the detection limit of the RNA-PCR, while the cells remain essentially viable and even active (Hammes et al., 2011). In addition, it is to be noted that, despite the labile nature of mRNA, false-positive signals from residual transcripts can occur in the case of high levels of dead bacteria ($>10^4$ cells ml^{-1}) (Sheridan et al., 1998; Vaitilingom et al., 2006).

An alternative approach to detect viable cells by PCR (herein referred to as v-PCR) was presented in 2003 by Nogva et al. (2003) by introducing the concept of EMA-PCR. The invention of the alternative molecule propidium monoazide (PMA) in 2006 (Nocker et al., 2006) similarly resulted in the term PMA-PCR. The distinction between viable and non-viable cells for both membrane impermeant dyes is based on membrane integrity. Microbiological samples are treated with a nucleic acid intercalating dye that selectively enters cells with compromised cell membranes, whereas an intact cell membrane presents a barrier for this molecule. Once inside a (dead) cell, the dye intercalates into the cell's DNA to which it is believed to covalently crosslink after exposure to strong visible light due to the presence of an azide group. Photolysis converts the azide group into a highly reactive nitrene radical (DeTraglia et al., 1978), which can react with any organic molecule in its proximity. Reaction with DNA can be assumed to occur with a high probability considering the spatial proximity of the intercalated dye. The modification was empirically found to strongly inhibit its amplification (Nocker and Camper, 2006; Rudi et al., 2005a). At the same time when the cross-linking with DNA occurs, any unbound excess dye reacts with water molecules. The resulting hydroxylamine (Graves et al., 1981) is no longer reactive, preventing reaction of the dye with DNA extracted from intact cells (Nocker and Camper, 2009).

Viable PCR is a promising technique because it makes use of the speed and sensitivity of the molecular detection while at the same time providing viability information. EMA and PMA have since their invention been applied to a wide variety of microorganisms including bacterial vegetative cells (Agusti et al., 2010; Bae and Wuertz, 2009; Cawthorn and Witthuhn, 2008; Delgado Viscogliosi et al., 2009; Nocker et al., 2009; Pan and Breidt, 2007; Rudi et al., 2005a; Soejima et al., 2007), bacterial spores (Rawsthorne et al., 2009), fungi (Vesper et al., 2008), viruses (Fittipaldi et al., 2010; Graiver et al., 2010; Sanchez et al., 2012), yeast (Andorrà et al., 2010; Shi et al., 2012), and protozoa (Brescia et al., 2009; Fittipaldi et al., 2011a). The addition of a pre-treatment step to the sample analysis to inhibit the amplification of DNA from membrane-damaged cells has been used in combination with end point PCR (Brescia et al., 2009), real-time or quantitative PCR (qPCR) (Rudi et al., 2005a), reverse transcription PCR (Graiver et al., 2010), isothermal amplification (Chen et al., 2011; Lu et al., 2009; Wang et al., 2012), denaturing gradient gel electrophoresis (DGGE) (Nocker et al., 2007a), terminal restriction fragment length polymorphism (TRFLP) (Rogers et al., 2008), microarray technology (Nocker et al., 2009), and next-generation sequencing (Nocker et al., 2010). Apart from providing evidence of feasibility, the research has illustrated the urgent need for adding viability information to DNA-based detection methods in diverse fields ranging from testing of food and water safety to clinical microbiology.

Despite its advantages, there is evidence demonstrating that v-PCR using DNA-intercalating dyes has practical and theoretical limitations especially when applied to environmental samples (Pisz et al., 2007; Varma et al., 2009; Wagner et al., 2008). Apart from sample-specific challenges, the two dyes both seem to have specific advantages and disadvantages. Whereas the greatest concern with EMA lies in its lack of specificity for intact bacterial cells, the greatest concern with PMA is the generation of false-positive signals due to incomplete signal suppression. For a successful application of v-PCR a couple of factors have to be considered that can influence the outcome of the resulting data, including the choice of the dye, its concentration, the incubation conditions, the light source, the presence of a high number of dead cells; the presence of high levels of suspended solids or biomass in the analyzed samples; the salt concentration in

the reaction mix; the pH of the reaction mix; the length of the target gene; and the sequence of the target gene among others. This review aims at discussing these parameters and their potential implications to increase the chances of generating specific, reproducible, sensitive, and accurate data. We hope that consideration of the critical factors in the experimental design ultimately increases the chances of integrating viability dyes in routine diagnostics.

2. Interaction of viability dyes with DNA and mechanism of amplification signal suppression

2.1. Comparison of photoreactive dyes with their non-reactive analogs

Ethidium bromide (EB) has been used extensively as a DNA-intercalating agent (Yielding et al., 1984). Several studies have shown that its photosensitive analog, EMA, intercalates into the DNA double helix similarly to the parent compound (Bolton and Kearns, 1978; Garland et al., 1980; Graves et al., 1981; Yielding and Firth, 1980). Unlike that in EB, interactions between EMA and nucleic acids are rendered irreversible through covalent attachment of the drug in situ (White and Yielding, 1977). The non-covalent interaction of EMA with DNA was shown to be essentially identical with that of the parent EB and to be primarily intercalative in nature. However, the DNA interaction with the diazide, apparently a stacking interaction, is quite different and much less active than the one of EMA and EB (Graves et al., 1981).

The binding of ethidium to DNA has been described to proceed in two distinct steps — an initial electrostatic interaction between the negatively charged phosphate oxygen atoms and the positively charged phenanthridinium ring nitrogen atoms, followed by intercalative stacking interactions between bases that stabilize the ligand–DNA complex through hydrophobic interactions (Garbett et al., 2004). At high ligand concentrations, a secondary binding mode was observed involving 'external' stacking of ethidium molecules in the DNA grooves resulting from ionic interactions with the polyphosphate backbone (Laugaa et al., 1983; Waring, 1965).

PMA, on the other hand, is identical to propidium iodide (PI) except that the additional presence of an azide group allows crosslinkage to DNA upon light exposure (Nocker et al., 2006). PI is the dye that has been most extensively used to identify dead cells in mixed populations because it is highly membrane impermeant and generally excluded from intact cells. Upon penetrating membrane-compromised cells, PI is capable of forming at least two binding complexes with nucleic acids. One binding involves PI intercalation between the bases with little or no sequence preference and with a stoichiometry of one dye molecule per 4–5 base pairs of DNA (Waring, 1965; Barni et al., 1981). The other binding consists in the electrostatic interaction with phosphate groups of single-stranded nucleic acids (Barni et al., 1981).

Although the two dyes behave nearly identically as intercalating stains for double-stranded nucleic acid, they differ in regard to their permeation through cell membranes. Ethidium enters intact cells (Nebe-von-Caron et al., 2000) suggesting that extreme care must be taken when interpreting the uptake and exclusion of EB-based dyes. An intact cell membrane on the other hand poses an efficient barrier for PI (and probably PMA) (Shapiro, 2003). This is in agreement with the idea that EB and PI are not interchangeable. Influx of EB is believed to be a passive process driven by the concentration gradients alone (Jernaes and Steen, 1994). The efflux systems of the cells can play a role in the EMA accumulation inside the cell. Intracellular concentration of a given compound is, generally, a result of interplay between permeability and efflux (Nikaido, 2001). Active efflux systems can provide resistance by extruding noxious compounds prior to reaching their target (Rodriguez et al., 2011). EB is widely used as an efflux pump substrate to detect and quantify efflux activity by bacteria (Greulich, 2004; Schumacher et al., 2007), and EMA could exert a similar effect on microbial cells (Flekna et al., 2007). However, the study by Rudi et al. (2005a) indicates that the exclusion of EMA from viable cells appears to be a passive process through diffusion barriers and not an active pumping process. The mode of exclusion of PMA from viable cells is still not clear.

2.2. Mechanism of amplification signal suppression

The mode of action of EMA/PMA in the DNA amplification signal is still not completely understood. Once inside the cells, they intercalate with a certain stoichiometry into nucleic acids. The azide groups that both dyes have in common, allow in theory covalent crosslinkage of the dye to the DNA upon light-exposure. Such a binding event can in theory be assumed to inhibit DNA amplification. One study suggested that upon binding, photo-induced cross-linkage might render the DNA 'insoluble' resulting in its loss during the DNA extraction procedure together with cell debris (Nocker and Camper, 2006). The study was however based on disappearance of dye-modified DNA bands on agarose gels and did not sufficiently account for the interference of dye binding with visualization with ethidium bromide staining. It is still conceivable that the overall reduction of the negative charge of DNA due to the addition of positively charged dye molecules could interfere to some extent with its electrostatic interaction with silica resin that is commonly employed for DNA purification. A more likely explanation however is that binding of the dye results in DNA fragmentation: Soejima et al. (2007) have reported that treatment with EMA followed by visible light irradiation directly cleaves the chromosomal DNA of dead bacteria. The hypothesis was nicely supported by electron microscopic pictures showing different degrees of DNA fragmentation upon exposure to increasing concentrations of EMA. Fragmentation would for obvious reasons interfere with amplification. Nevertheless, the questions about how efficiently the dyes bind and the impact of binding on DNA structure and function have still not been answered. Yielding et al. (1984) pointed out that in the formation of DNA-adducts the strong association alone is not sufficient to provoke an appropriate change in the properties of DNA because of complex factors such as drug distribution and metabolic disposition.

3. Specificity of viability dyes for live and dead cells

Treatment of microbial mixtures containing live and dead cells should ideally lead to the exclusion of signals from dead cells, but should not affect signals from live cells. Both dyes show pronounced differences in the specificity for live cells.

3.1. Exclusion of viability dyes from live cells

Some studies have demonstrated that EMA may penetrate bacterial cells with intact membranes and cause underestimation of live cell population (Cawthorn and Witthuhn, 2008; Flekna et al., 2007; Kobayashi et al., 2009; Loozen et al., 2011; Nocker and Camper, 2006; Nocker et al., 2006; Rueckert et al., 2005). The extent of EMA uptake by intact cells was reported to depend on the bacterial species (Flekna et al., 2007; Nocker et al., 2006), the EMA concentration (Kobayashi et al., 2009; Meng et al., 2010; Wang et al., 2009), and the density of the bacterial suspension (Flekna et al., 2007; Kobayashi et al., 2009). For those species whose intact cell membranes are not sufficiently impermeable for EMA, the dye might be considered inadequate to differentiate between live and dead cells. PMA has been proposed as a more appropriate alternative with a substantially higher specificity for live cells. The increased specificity of PMA for live cells is thought to be mainly due the higher charge of PMA (EMA has one positive charge, and PMA has two) (Nocker et al., 2006). The following sections are dedicated to the three distinct

methods suitable to assess specificity: fluorescence microscopy, qPCR signal reduction, and cytotoxicity.

3.1.1. Fluorescence microscopy

Early evidence for the differences in specificities of the two dyes was provided in a comparative study by Nocker et al. (2006) applying the two dyes (50 μg/ml) to nine different bacterial species. Dye penetration was visualized by fluorescence microscopy. Results suggested that PMA was efficiently excluded from cells with intact cell membranes for all species studied (Nocker et al., 2006). EMA, on the other hand entered live intact cells within 5 min for six out of the nine species resulting in intense red staining (*Escherichia coli* O157:H7, *Listeria monocytogenes*, *Micrococcus luteus*, *Mycobacterium avium*, *Staphylococcus aureus*, and *Streptococcus sobrinus*). Only for *Salmonella typhimurium*, *Pseudomonas syringae* and *Serratia marcescens*, EMA treatment did not result in visible staining under the conditions used. The in part insufficient specificity of EMA was confirmed in later studies. Assessing the usefulness of the dye for *Campylobacter jejuni* and *L. monocytogenes*, Flekna et al. (2007) reported a dye concentration of 240 μM applied to a mixture of live and dead cells that bacteria were stained independently of their viability status (exposure time for 5 min at 4 °C). Lowering the EMA concentration resulted in less staining. The EMA threshold concentration below which substantially less staining was observed tended to be around 24 μM. Permeability studies of PMA (50 μM) and EMA (5 μM) through the membranes of live and dead *Helicobacter pylori* cells (exposure for 5 min) suggested that, under the study conditions, the membranes of live *H. pylori* effectively prevent penetration of PMA but allow the passage of EMA (Nam et al., 2011).

3.1.2. qPCR signal reduction

The majority of data relating to dye specificity was obtained by comparing qPCR threshold cycle values for cells treated with a viability dye or not. When exposing live bacterial cells to a dye, EMA tends to cause greater signal reduction (compared to controls that were not treated with dye) than PMA. As for the microscopic examination, signal reduction for live cells however is species-specific. Although PMA seems to be substantially more specific for live bacterial cells, modest increases in threshold cycle (Ct) values have also been reported when treating potentially live cells with this dye (Kralik et al., 2010; Liang et al., 2011; Loozen et al., 2011; Vesper et al., 2008; Yañez et al., 2011). Increases in the Ct value could to some extent indicate the presence of membrane-compromised cells in the "live" cell suspensions (Vesper et al., 2008; Yañez et al., 2011). While even pure cultures can be expected to contain a certain proportion of dead cells (especially in solid media), membrane permeability can also undergo changes in live cells dependent on the physiological status. When analyzing the influence of different growth stages of a pure *E. coli* culture treated with EMA, Gedalanga and Olson (2009) found that rapidly dividing and senescent cells were most affected by dye treatment. It was suspected that membrane permeability is affected by the cell wall composition which undergoes changes as the cells progress through different growth phases.

3.1.3. Cytotoxicity

Maybe the most sensitive method to study the impact of dyes on live cells is the measurement of cytotoxicity when exposing live cells to the dyes. The less colonies are obtained after exposure, the greater the cytotoxic effect and the less selective the dye. The concept of exposing live cells to a viability dye for cytotoxicity testing was first used by Rueckert et al. (2005). A suspension of live *Anoxybacillus flavithermus* (3.6×10^5 CFU ml^{-1}) was exposed to different EMA concentrations (0.1, 1, 10, and 100 μg/ml) for 5 min. The observed cytotoxic effect was concentration-dependent and was very minor at the lowest concentration, whereas 1 μg/ml EMA resulted in an approx. 2 log reduction in CFU numbers. Higher concentrations completely abolished growth. Findings have to be seen in a species-specific context: in a later study with *E. coli* by Soejima et al. (2007), cytotoxicity was only minor when exposing cells to an EMA concentration of up to 10 μg/ml for 3 h, whereas 100 μg/ml resulted in a 3 log reduction. In a study on *L. monocytogenes*, Pan and Breidt (2007) preconditioned mixtures of different strains for 2 h at different non-lethal temperatures (5, 15, 25, and 37 °C) followed by exposure to EMA (240 μM) or PMA (50 μM). Before plating samples were either light-exposed or not. For EMA, the cytotoxic effect (without light exposure) increased in a temperature-dependent manner with higher preconditioning temperatures yielding less colonies than lower temperatures. In combination with light exposure, on the other hand, EMA treatment resulted in complete elimination of growth independent of the precondition temperature. For both scenarios (without or with light exposure), only a very minor cytotoxic effect was observed for PMA independent of light exposure. The MICs of EMA and PMA were reported to be 240 and 1600 μM. A similar experiment was performed with *Legionella pneumophila* by Yañez et al. (2011). Aliquots of live *L. pneumophila* suspensions were preconditioned to different non-lethal temperatures (4, 22, 35, and 44 °C; for 2 h each) followed by treatment with 100 or 200 μM of either EMA or PMA and light exposure, respectively. *L. pneumophila* is known to survive temperatures below 60 °C for elongated time periods. Cells in thus treated suspensions were enumerated by plate counting and resulting numbers were compared to aliquots which had been exposed to the same temperatures, but not treated with any viability dye. Results indicated that 100 μM PMA treatment only minimally reduced colony counts (compared to the controls that were not treated with dye). Slightly higher cytotoxicity was observed at 200 μM with the strongest reduction in colony numbers (0.44 log units) with cells preconditioned at 44 °C. EMA treatment on the other hand resulted in a substantial cytotoxic effect ranging from 1.32 to 3.63 log units. The discrepancy between cultures treated with EMA or not was increasingly stronger with higher temperatures and higher dye concentrations. The result (similar to the one observed by Pan and Breidt (2007) for *L. monocytogenes*) indicated underestimation of live cell numbers due to penetration of EMA into these cells.

Flekna et al. (2007) reported in the above mentioned study with *C. jejuni* and *L. monocytogenes* that even at low EMA concentrations for which no staining was seen under the microscope, plate counting still revealed a substantial effect on cell viability. The cytotoxic effect was dependent on the EMA concentration. Using suspensions of live cells of 3.6×10^4 and 1.7×10^5 cells ml^{-1} of *C. jejuni* and *L. monocytogenes*, growth was completely abolished at EMA concentrations of 5 μg/ml (exposure time for 5 min at 4 °C). For denser cell suspensions of 2.2×10^8 (*C. jejuni*) and 2.4×10^9 cells ml^{-1} (*L. monocytogenes*), decreases in cell numbers of approx. 4 and 7 log units were observed for an EMA concentration of 10 μg/ml, respectively. For all cell suspensions, even an EMA concentration of 1 μg/ml resulted in about 1 log unit less growth.

3.2. Exclusion of dead cell signals

Whereas dye penetration into cells is not desired with live cells, efficient uptake by dead cells determines the efficiency to exclude false-positive signals in combination with amplification. Possible false-positive results, have been observed with different microorganisms (Bae and Wuertz, 2009; Fittipaldi et al., 2011a; Kralik et al., 2010; Soejima et al., 2011a). LØvdal et al. (2011) cautioned against the use of PMA-qPCR to discriminate between viable and dead *Listeria innocua* cells because it underestimated heat-killed cells in comparison with plate counts and fluorescence microscopy. The same properties that prevent entry of PMA into live cells, seem to reduce the penetration of the molecule into dead cells in comparison with the more membrane-permeable EMA. When comparing survival of a mixed bacterial population from cod filets subjected to different temperatures from 45 to 95 °C (for 5 min each) and treated with low concentrations of EMA (1 μg/ml) or PMA (3 μg/ml), bacterial numbers

determined by PMA-qPCR were found to be higher than the ones determined by cultivation techniques or EMA-qPCR (Lee and Levin, 2009a). The finding suggests that EMA penetrated heat-exposed cells more effectively than PMA resulting in more efficient inhibition of amplification of DNA from heat-damaged cells by EMA. A more efficient penetration of EMA into membrane-compromised cells was also observed by other authors (Chang et al., 2010; Chen and Chang, 2010). When treating dead *Legionella* cells (heated at 95 °C for 2 min or exposed to 0.5–1 ppm of chlorine for 30 min) with EMA or PMA, a 4-fold higher dye concentration was required for PMA in comparison with EMA to obtain the same level of signal reduction of 5 log units (Chang et al., 2010). Comparing the same dye concentration, the maximal signal reduction with killed cells was 0.5–1 log units higher for EMA than for PMA (although the latter had an advantage of lowering the signals from live cells to a lesser extent). The obviously higher capacity of EMA to minimize false-positive signals is probably due to a more efficient penetration of the dye into membrane-compromised cells resulting in higher intracellular concentrations, increased saturation of DNA, and thus a stronger inhibition of PCR amplification (Yañez et al., 2011). The only study where EMA failed to suppress signals from dead cells was by Rawsthorne et al. (2009). In contrast to PMA, addition of EMA to heat-inactivated *Bacillus subtilis* spores (killed by double autoclavation at 121 °C for 20 min each) did not result in qPCR signal suppression. Application of viability dyes to bacterial spores does however need confirmation due to the lack of follow-up studies which is surprising given the relevance for the food industry.

The exclusion of dead cell signals is even more challenging when working with environmental samples. The viability PCR did not reflect a comparable reduction in the numbers of live cells in comparison with culture techniques when applied to wastewater (Varma et al., 2009), anaerobic-fermentor sludge samples (Wagner et al., 2008), elemental sulfur samples, biofilm samples (Pisz et al., 2007), or to environmental water samples (Qin et al., 2012). The differences in the signal reduction varied and ranged between 2 and 4 log units. Therefore the use of v-PCR to differentiate between viable and non-viable organisms appears to be matrix dependent. The relevance of the different factors related with the sample matrix complexity is discussed in the following sections of this review. However, it is important to note that in some cases enumeration of microorganisms using v-PCR was similar to culture in certain wastewater (Bae and Wuertz, 2009; Gedalanga and Olson, 2009) and soil samples (Pisz et al., 2007; Taskin et al., 2011) suggesting that this technique can in principle be used in environmental samples.

3.3. Potential improvements to increase specificity

Despite its slight advantage of stronger suppression of dead cell signals, the usefulness of EMA can be severely hindered by the penetration of intact cells in a species-dependent manner. PMA, on the other hand, proves more selective for live cells, but could show a greater suppression of dead cell signals. Different approaches have been chosen to overcome these deficiencies.

3.3.1. EMA

The main strategy to decrease uptake of EMA by live cells was to lower the concentration of the dye resulting in less dye molecules entering live cells. The approach is made possible by EMA's efficiency to penetrate dead cells even at relatively low dye concentrations. Whereas early studies employing EMA typically used final dye concentrations of 100 μg/ml (238 μM) (Douglas Inglis et al., 2010; Flekna et al., 2007; Graiver et al., 2010; Marouani-Gradi et al., 2010; Nocker and Camper, 2006; Rudi et al., 2005a), concentrations in more recent studies were chosen substantially lower. Cawthorn and Witthuhn (2008) have found that the use of three different EMA concentrations with viable *Enterobacter sakazakii* cells — 100 (238 μM), 50 (119 μM), and 10 μg/ml (23.8 μM) – resulted in very different PCR product yields of 74, 82 and 92%, respectively (compared with 95, 96, and 97% after PMA treatment). A concentration of 10 μg/ml EMA was found to be suitable in various studies (Minami et al., 2010; Shi et al., 2011; Soejima et al., 2011a; Wang et al., 2009), whereas a higher concentration resulted in penetration of live cells. Minimum amounts of EMA required for effective signal suppression from dead cells on the other hand were reported to be 2.5 μg/ml for *Vibrio vulnificus* (Wang and Levin, 2006), 2.3 μg/ml for *Legionella* (Chen and Chang, 2010), 1.5 μg/ml for *Bifidobacterium* (Meng et al., 2010), and 1 μg/ml for bacterial flora from fish filet (Lee and Levin, 2009a). It has to be considered that published values greatly depend on experimental conditions such as killing conditions, the purity of the cell suspension, the type of light source and exposure time. Apart from minimizing EMA concentration, an increasing number of studies reported dye incubation on ice or low temperature (4 °C) instead of room temperature (see section Incubation temperature). As temperature is a well-known determinant of membrane permeability (van de Vossenberg et al., 1995), this strategy could be useful for minimizing EMA uptake by live cells.

3.3.2. PMA

The main strategy to improve the exclusion of dead cell signals using PMA has been the amplification of longer DNA sequences (see separate section). Other potential strategies might consist in the treatment of samples with modest membrane-destabilizing agents and sample exposure at higher temperatures. Application of higher dye concentrations can also be considered. For instance, high PMA concentrations such as 100, 150, and 200 μM have been used without significant cytotoxic effects on viable cells for *Bacteroides* (Bae and Wuertz, 2009), *Cryptosporidium* oocysts (Brescia et al., 2009), and protozoa (Fittipaldi et al., 2011a).

3.4. Summary of dye specificity characteristics

Based on all this information, both EMA and PMA may be effectively used in the v-PCR method as intercalating dyes. However, several studies have showed that EMA is slightly more efficient in signal suppression than PMA, but PMA is more effective than EMA in terms of live-dead distinction. Taking this into account, optimization of EMA concentrations should focus on minimizing dye uptake by intact cells, whereas for PMA efforts should concentrate on maximizing suppression of dead cells signals. It should be noted, however, that these recommendations currently refer mainly to bacteria, which have been used in the majority of studies. Different findings might apply to viruses and fungi. Andorrà et al. (2010) did not observe differences between the two dyes when quantifying live yeast cells in wine. Interestingly, the optimum concentration for EMA was found to be higher (24 μM) than the one for PMA (6 μM) in the case of yeast. It seems advisable to determine optimal dye concentrations that efficiently inhibit DNA amplification from membrane-compromised cells, while not affecting signals from intact cells.

4. Dye incubation conditions

Apart from the dye concentration, dye incubation time and incubation temperature can be considered as the most important parameters and are discussed in the following. All parameters are interlinked requiring an optimized balance for the particular application of interest. In addition light conditions in the light exposure step have to be taken into consideration. Cells are typically exposed to the viability dye in the dark due to the photoreactive nature of EMA and PMA. Avoiding light exposure might be particularly important once the dyes are added to cell suspensions and greatly diluted. For concentrated stock solutions, temporary light exposure might not be an overly critical factor. When exposing a 20 mM PMA solution, the dye was still found to be functional after a 2 min exposure to a 650 W halogen lamp which was

typically used for experiments (unpublished data from Andreas Nocker), the reason being most probably a very limited light penetration due to efficient light absorption on the surface leaving the majority of the dye molecules reactive. Dilution of the dye to the final concentration, once added to a cell suspension, on the other hand, renders the molecules highly susceptible to light activation given the reactive nature of the azide group. Potential degradation products of PMA could comprise EB and PI in addition to unidentified intermediate products as indicated by HPLC analysis of dye exposed to laboratory light for 1 h (unpublished data from GenIUL S.L. (Barcelona, Spain)).

4.1. Incubation time

The time that cells are exposed to a viability dye has to be sufficiently long to allow the chemical to enter membrane-compromised cells and to intercalate into their DNA. Incubation times have to be seen in context with the targeted microbial species and the applied dye concentration. As a rule of thumb, more care is required with high dye concentrations (especially with the more membrane-permeant EMA) to prevent the dye from penetrating intact cells so that it does not produce false-negative results. Using low dye concentrations, incubation times can be considered more flexible. Exposing *Enterobacteriaceae* (live and dead) to a final concentration of 10 μg/ml EMA, no significant differences in v-PCR results were obtained with incubation times of 10, 20, 30, and 60 min (Soejima et al., 2011a). For bacterial studies, an incubation time of 5 min is generally accepted and most commonly used (Cawthorn and Witthuhn, 2008; Chang et al., 2010; Chen and Chang, 2010; Gedalanga and Olson, 2009; Graiver et al., 2010; Kobayashi et al., 2009; Kramer et al., 2009; Nocker et al., 2006; Nocker et al., 2007b; Pisz et al., 2007; Soejima et al., 2008; Varma et al., 2009; Wang and Levin, 2006). When studying the effect of PMA exposure time (1, 5, and 15 min) on signal suppression (using isopropanol-killed *E. coli* O157:H7 or *S. sobrinus*), a substantial signal reduction was already measured after 1 min of exposure (Nocker et al., 2006) making 5 min appear an appropriate time period even for more complicated matrices. Longer exposures of up to 60 min did not result in increased efficiency (personal communication). Similar results were reported for *Cryptosporidium parvum* oocysts treated with 150 μM PMA (Brescia et al., 2009). Despite the fact that the dye has to cross several complex barriers (oocysts wall, sporozoite cell and nuclear membranes), an incubation time of 5 min was surprisingly found sufficient to enter heat-killed oocysts (70 °C, 30 min), whereas viable oocysts were not affected by the treatment even after 30 min. For *Acanthamoeba castellanii* (both trophozoites and cysts) an incubation time of 30 min was reported (Fittipaldi et al., 2011a). For yeast species, that are typically found in wine, a study by Andorrà et al. (2010) found that 10 min was optimal for both EMA and PMA, when comparing different incubation times (5, 10, 15, 20, and 30 min), whereas a PMA incubation time of 20 min was reported for fungal cells added to a filter (Vesper et al., 2008). Long incubation times (50 min) were also reported for bacterial spores (Rawsthorne et al., 2009). An alternative to a prolonged dye incubation time in the case of organisms with low dye penetration rates, repeated dye treatment (with intermediate light exposure, harvesting, and resuspension of treated sample prior to the next treatment) might be useful. For *Mycobacterium paratuberculosis*, a double exposure to 25 μM PMA for 5 min each with intermediate light exposure was found to be more beneficial than a 50 min single dye incubation step (Kralik et al., 2010). Double treatment with a low dye concentration also provided good results in the case of EMA treatment of *E. sakazakii* (Minami et al., 2010).

4.2. Incubation temperature

As temperature is a well-known determinant of membrane permeability (van de Vossenberg et al., 1995), it can be considered an important experimental parameter. Incubating samples with viability dyes at low temperatures might prove especially useful at minimizing EMA uptake to live cells given the lower specificity of this dye in comparison to PMA. First experiments looking at the effect of different temperatures were based on pre-conditioning pure cultures to different sublethal temperatures prior to cooling to room temperature and addition of EMA or PMA (Pan and Breidt, 2007; Yañez et al., 2011). In comparison with a control sample without viability dye and in comparison with culture results, EMA uptake was greatly diminished at lower temperatures, whereas higher temperatures resulted in false-negative results due to unspecific dye uptake by live cells. The temperature effect was substantially lower with PMA due to its lesser membrane permeability. Although cells were exposed to the different temperatures prior to dye addition and dye incubation was performed at room temperature, results indicate that membrane fluidity might still have been altered by the preconditioning temperature. The findings underline the importance for further research on the effect of temperature, which might result in new possibilities of optimization. Whereas most studies have used room temperature while exposing samples to viability dyes, a number of newer studies reported EMA incubation at low temperatures (4 °C or ice). Despite the lack of a direct comparison of different EMA incubation temperatures, good results were obtained by Chang et al. (2009, 2010), Soejima et al. (2008, 2011a), and Minami et al. (2010) when treating *L. pneumophila*, *L. monocytogenes*, *Enterobacteriaceae*, and *E. sakazakii* with 10–20 μg/ml EMA at 4 °C. Given the undefined nature of dye uptake, both a passive transport and facilitated diffusion (mediated by channels or carriers) through the membrane are conceivable. Passive diffusion would underlie the greatest temperature dependence as cellular membrane fluidity is strongly influenced by temperature. Assuming diffusion, lower incubation temperatures would therefore be greatly beneficial to avoid EMA accumulation in live cells. More insight into the temperature dependence can be assumed to contribute to understanding the mechanism of uptake and to differentiate between passive transport, channel or carrier facilitated diffusion characterized by different temperature dependences.

5. Photoactivation

5.1. Light source

Initial research with viability dyes employed photoactivation of viability dyes using high-power halogen lamps (500–750 W) developed for stage lighting. These lamps are currently still used and are functional for research purposes although different lamps differ in their efficiency for light activation, probably due to different emission spectra. A lamp (or more precisely the bulb) can be considered suitable if the spectrum has a high proportion of the wavelengths necessary for photoactivation. Absorption maxima are 456 nm for EMA and 464 nm for PMA. Samples are typically placed horizontally on ice with the most transparent side facing up at a distance of 20–30 cm from the light source (Cawthorn and Witthuhn, 2008; Meng et al., 2010; Nocker and Camper, 2006; Pan and Breidt, 2007; Soejima et al., 2008; Wang and Levin, 2006). During exposure it is advised to mix samples by tilting the ice container to guarantee homogeneous exposure of the sample both to the dye and to the light. Ice can be seen to be advantageous both by preventing excessive heating of the samples (halogen lamps emit a substantial amount of heat) as well as by maximizing light exposure due to its reflective properties. A disadvantage of the procedure using halogen lamps can be seen, apart from the intense emission of heat, in experimental variation caused by the manual procedure. Differences in exposure or mixing efficiency can be expected to result in experimental variation and lower reproducibility between assays. Indeed it is difficult to cross-compare results from different studies given the wide variety of lamps and differences in exposure procedures.

An alternative to halogen lamps is the use of light-emitting diodes (LEDs), which were first introduced for this application by Vesper et al. (2008). The emission of blue LEDs at around 465 nm is nearly identical with the absorption maximum of PMA (464 nm) and EMA (456 nm). Whereas Vesper et al. (2008) used an array of LEDs to expose PMA-treated cells collected on a filter, a later commercial LED-based instrument was designed for light exposing cell suspensions (PhAST Blue, GenIUL, Barcelona, Spain). Up to 12 microcentrifuge tubes can be processed simultaneously. Functionality was successfully demonstrated in studies by Agusti et al. (2010), Fittipaldi et al. (2010, 2011a), Elizaquivel et al. (2012), Miotto et al. (2012), and Sanchez et al. (2012). In comparison with halogen lamps, LEDs have a decisive advantage in emitting light of a defined specific wavelength (in this case in the blue range of the visible spectrum) allowing for optimal dye activation and avoiding the generation of heat. Comparing different EMA treatment procedures with and without ice on live *E. coli* O157:H7 cells, Wang et al. (2009) reported better replicability and a lower standard deviation when a cooling step was involved. The omission of cooling and the more homogeneous sample processing result in increased simplicity and user-friendliness, while at the same time reducing experimental variation. The implementation of LED technology can thus be seen as an important step in the standardization of photoactivation.

5.2. Light exposure time

Light exposure of dye treated samples is important for (i) activation of nucleic acid-bound dye and for (ii) inactivation of excess dye that has not entered cells and that could potentially bind to DNA from live cells during the DNA extraction procedure. Whereas in the first case the dye molecules react with DNA, the reaction in the second case occurs with water leading to the formation of hydroxylamine that can no longer covalently crosslink. Both processes have to be highly efficient to guarantee reliable PCR results. Considering the great diversity of light sources used in different studies it is not surprising that the literature does not give consistent information in regard to the optimal light exposure time. The times required for optimal assay performance were typically reported to be in the range between 2 and 20 min. As different lamps have different spectra and emit different amounts of the wavelength required for photoactivation, a large portion of this discrepancy might be attributed to different light sources. Other reasons for varying optimal light exposure times could lie in varying distances of the sample from the light source (determining the light intensity), different sample turbidities (caused by abiotic and biotic components or the cells themselves) or the characteristics and light absorbing properties of the sample matrix. In pure water, complete photolysis was reported for EMA in 30 s (Nogva et al., 2003).

When optimizing light exposure time with a given light source, experiments should be performed both with dead and live cells. Whereas the exposure time has to be long enough to ensure maximum suppression of dead cell signals, it should not result in underestimation of live cell numbers. When examining the end-point PCR products of PMA-treated oocyst samples (using an 800 W lamp), light exposure times of 1 min and higher were reported to prevent amplification of target sequences (partial *hsp70* and SSU rRNA genes) from heat-killed oocysts (Brescia et al., 2009). Band intensities of PCR products (346 bp for hsp70 and 834 bp for the SSU rRNA gene) from live oocysts were comparable with the ones from the non-PMA treated control for light exposure times of up to 4 min, whereas 5 min resulted in a slight reduction of the shorter of the two PCR products (for an explanation we refer to the section about the effect of amplicon length). The effect of light exposure time on live cell signals could possibly be caused by excess heat generated by halogen lamps, which may render cell membranes permissive for dye penetration by inflicting membrane damage (Wang et al., 2009).

Light exposure times can further be assumed to differ between LED and visible light sources. Intense visible light was shown to induce DNA damage like single-strand breaks and other DNA modifications in mammalian cells (Pflaum et al., 1994) suggesting the necessity for avoiding excess light exposure. Wavelengths between 400 and 500 nm were reported to be responsible for most DNA alterations. Additionally the light dose received by the sample is strongly related with the microtube material, in some cases different materials show differences of up to 50% in transmittance in the wavelength range relevant for activation of viability dyes (data not shown). Li and Chen (2012) compared different transparent containers for the photoactivation step; and they found that none of the microtubes tested allowed them to achieve the desirable cross-linking effect. Taking into account the possible effect of the tubes in the photoactivation step, the microtube used in the v-PCR should be standardized.

6. Target gene

6.1. Length of the target gene

The importance of choosing the right v-PCR amplification strategy is gaining increasing attention with the length of the amplified target gene being an important parameter determining the outcome of signal suppression from dead cells. The underlying principle is that viability dyes once they enter membrane-compromised cells bind to DNA in a certain stoichiometry. The damage (e.g. DNA cleavage) inferred to the DNA upon light exposure interferes with its amplification. In theory one modification in the targeted DNA is sufficient to suppress the amplification of the particular template. As a consequence, the amplification of longer DNA sequences correlates with a higher probability that the DNA polymerase encounters such a modification in the stretch targeted by the primers resulting in a stronger suppression of signals from membrane-compromised cells (Contreras et al., 2011; Soejima et al., 2008; Soejima et al., 2011a). An effect of amplicon length on PCR amplification in v-PCR can always be expected when the saturation of DNA with dye molecules is not complete in the amplified region for the entire population of template molecules, or in other words if not all DNA templates are affected by the dye treatment. For EMA when used at the ng ml^{-1} concentrations, a stoichiometry of one molecule binding to 10–80 bp had been reported in vitro (Marx et al., 1997). Whereas no data is available for PMA, excess PI was estimated to reach a maximal binding stoichiometry of one dye molecule per 4–5 bp when added in 5 μg/ml to freely accessible DNA (Barni et al., 1981). Due to its structural identity, a similar value can be assumed for PMA, however such saturation levels are highly unlikely in the case of microorganisms where DNA is located within an envelope and is far from being freely accessible. Judging from the background amplification of dye-treated dead cell samples, the intracellular dye concentration within membrane-compromised cells and the resulting DNA binding frequency in vivo can be assumed to be substantially lower.

The first indications that amplicon length affects v-PCR results were obtained by Soejima et al. (2008) in a study reporting more efficient suppression of PCR amplification when targeting an 894 bp fragment of heat-killed *L. monocytogenes* compared with a 113 bp fragment. The same observation held true for PMA when analyzing PCR products from killed cells by denaturing gradient gel electrophoresis (Luo et al., 2010). Whereas dead cell signals were not efficiently suppressed by direct PCR amplification of a 190 bp fragment, this was achieved by using a nested PCR approach with the first PCR targeting a 1500 bp fragment followed by the amplification of a shorter fragment. And applying EMA and PMA to heat-killed *L. pneumophila*, a significant difference in the number of dead cells was obtained depending on whether qPCR was based on amplification of 16SrRNA (454 bp) or 5SrRNA (108 bp) (Chang et al., 2010). Loozen et al. (2011) found that for *Aggregatibacter actinomycetemcomitans* qPCR

targeting a longer DNA fragment (200 bp) resulted in an additional log decrease compared with qPCR amplifying a shorter DNA fragment (82 bp). Similar observations were made by some authors of this review applying PMA to heat-killed *L. pneumophila* cells at a final concentration of 50 μM. Comparing PMA-treated with untreated cells, signal reductions were 5.8, 7.9, and 8.1 cycles were obtained for amplicon lengths of 63, 127, and 148 bp (targeting the mip gene; unpublished results). Banihashemi et al. (2012) found that the PMA-PCR method amplifying a long amplicon reduced the dead cell signal in more than 7 log units for heat killed *Salmonella enterica* (1641 bp) and *C. jejuni* (1512 bp) cells. However a reduction of 3 and 4 log units was achieved when shorter DNA fragments of the same genes were used to amplify DNA from *S. enterica* (119 bp) and *C. jejuni* (899 bp) dead cells respectively. Non significant reductions in the live cell signals were observed when long DNA fragments were amplified. Martin et al. (2012) have used three different specific PCR targets differing in length (95, 285, and 417 bp) to detect and enumerate viable *Salmonella* cells in the presence of a high number of dead cells (up to 108 CFU/g) in cooked ham. They found that the inhibition effect was dependent on the PCR amplification product length, and only the longer product achieved suppression of 108 CFU/g of heat-killed cells. In the light of these findings, targeting longer sequences has the ability to overcome insufficient suppression of signals from membrane-compromised cells as reported in some previous studies (LØvdal et al., 2011; Rudi et al., 2005a,b).

A significant step towards more efficient exclusion of dead cell signals has recently been made by Soejima et al. amplifying long stretches of PCR in a quantitative manner (Soejima et al., 2011a). Comparing the degree of PCR inhibition after EMA treatment of dead cells, amplification of PCR products (16S and 23S rRNA genes) of increasing length from 110 bp to 2840 bp showed increasingly strong suppression with inhibition rates being proportional to the amplicon length. In the case of the longest amplicons of 1490 and 2840 bp, complete suppression of signals from 10^8 cells ml^{-1} was obtained. The authors concluded that despite the potentially poor efficiency of qPCR targeting long DNA sequences and lower detection limit, the approach in combination with EMA treatment is a promising tool for selective detection of live cells in a background of high levels of dead bacteria. As the study was performed with cells killed in a boiling water bath for 2 min (which can result in cell lysis), it still needs to be investigated whether complete signal suppression is also obtained with less severe killing conditions.

Similar results in regard to the effect of amplicon length were obtained in another study by the same authors (Soejima et al., 2011b). A real-time PCR mastermix was introduced allowing for the amplification of a long 16S rRNA gene sequence of 1514 bp leading to complete suppression of dead cell signals within 50 cycles. Although the study was performed on *Enterobacteriaceae* (as contained in milk), the approach can be seen as highly valuable for quantitative detection of microbial pathogens, but suffers from the limited availability of commercially available pathogen-specific primers that would allow for the amplification of comparably long DNA sequences. The fact prompted the authors to introduce a nested PCR approach (similar to the one described by Luo et al., 2010). Whereas the first PCR is based on the amplification of a long PCR product, a second PCR can make use of commercial kits based on pathogen-specific primers. The length of the first PCR amplicon ensures the efficient signal suppression of EMA-treated dead cells, whereas the 2nd PCR allows for specific detection of target organisms. The disadvantage of the approach is that the dilution of the 1st PCR product (which is required for complete dead cell suppression) and the nested approach per se result in a loss of the quantitative character of detection. Nevertheless we agree with the authors that the strategy can still be considered highly valuable for hygiene testing, but we would like to add that in the case of a more membrane-permeant EMA, the dye concentration has to be sufficiently low to avoid penetration of live cells as amplifying longer stretches of DNA equally increases the likelihood of underestimating live cell numbers. Comparing Ct values from EMA-treated live cells with the ones from non-EMA exposed live cells from different species, the signal reduction was not greater than 2 cycles for amplicon lengths of up to 1490 bp, whereas amplification of PCR products of 2840 bp resulted in signal reduction of >4 cycles (Soejima et al., 2011a). The greatest signal reduction was found for *Salmonella enteritidis* with a difference of 5.5 cycles between live cells treated with EMA or not.

Applying PMA to cells subjected to a heat stress gradient of increasing severity and amplifying DNA sequences of different lengths (from 140 to 724 bp) by qPCR, Contreras et al. (2011) observed that the effect of amplicon length on quantitative vPCR is dependent on the stress intensity. Whereas at low temperatures no mentionable effect of amplicon length was observed, the impact was increasingly stronger for higher temperatures. Interestingly, the relative difference in signal reduction when amplifying PCR products of different lengths varied along the stress gradient. Using the example of *Vibrio anguillarum*, a relative difference in signal reduction of only approx. 1 cycle was observed between the shortest and longest amplicon for cells exposed to 70 °C, whereas the difference increased to nearly 10 cycles when applying PMA-qPCR to cells exposed to 80 °C. Both temperatures can be considered lethal (as confirmed by absence of growth), although different degrees of membrane damage can be assumed. The correlation between the extent of membrane damage, signal reduction and amplicon length deserves further study.

6.2. Target gene sequence

Another influential factor in the non complete amplification signal suppression from dead cells when v-PCR is performed could be the sequence of the target gene. Chang et al. (2010) mentioned that besides the length of the target gene the DNA sequence like other possible factors influenced their results when two different genes of different lengths were used to detect viable *Legionella* cells.

Quantification of [^{3}H] EMA bound to various restriction enzyme fragments indicated that the drug appeared to preferentially bind at a G–C base pair in about half of the fragments. The other fragments were located in specific map regions of the plasmid and did not bind the drug with a strict dependence on G–C content suggesting that binding specificity may depend on more than one structural feature of the DNA (Hardwick et al., 1984). For propidium iodide, little or no sequence preference was reported, however, having one or two G · T base pairs at a site makes intercalation significantly less favorable (Wilson et al., 1986).

EMA cleavage directly took place without the assistance of DNA gyrase/topoisomerase IV and the DNA repair enzymes of bacteria, medium/high concentrations of EMA (1 to 100 μg/ml) led to breaks of double-stranded DNA and low concentrations of EMA (10 to 100 ng ml^{-1}) generated a single-stranded break (Soejima et al., 2007). Photoaffinity labeling of synthetic DNAs with EMA was studied to determine if the efficiency of adduct formation was related to DNA sequence (Dannelley et al., 1986). The results showed that there are significant differences in efficiencies of EMA adduct formation between DNA polymers of different sequences; being the drug much effective in photolabeling DNAs containing G–C pairs. However, the origins of the base pair effect may result from conformational features of the intercalation site and internal motions of the particular helix (Dannelley et al., 1986). The amount of drug covalently bound to a site is the result of a complex set of factors, which include at least the binding affinity of the drug and the photochemical efficiency of the reaction of the nitrene with a particular sequence (Dannelley et al., 1986). Additionally, the latter factor could be easily affected by adjacent sequences and structures. Whereas, the former factor could be affected by the mode of EMA binding to DNA, since besides the intercalation, a secondary binding process may arise from self-aggregation

of the ethidium cation along the negatively charged phosphate backbone of DNA (Blake and Peacocke, 1968).

Based on the aforementioned studies, and although experimental evidence remains to be provided, the contribution of sequence-dependent differences in the covalent adduct formation could be an important factor to take into account in the optimization of v-PCR method. DNA sequence could make drug intercalation more favorable or less. That might explain the observation that different genera of *Enterobacteriaceae* appeared to demonstrate different cross-linking and cleavage rates with EMA for the same gene length of PCR amplicons (Soejima et al., 2011a).

7. Species-specific differences in vPCR

Various studies applying the same experimental conditions to different species described species-specific differences. For live cells, a direct microscopic comparison of different species exposed to EMA and PMA revealed a much greater heterogeneity for dye uptake for EMA, whereas no or very minimal staining was observed for PMA for the incubation time of 5 min (Nocker et al., 2006). PMA treatment might therefore provide more comparable results when applied to cells of different microbial species or samples of mixed microbial composition. With differences in membrane permeabilities of the dyes being a relatively simple explanation for live cells, the situation in regard to dead cells is more complex. When applying EMA-qPCR to different heat-killed enterobacteria, Soejima et al. (2011a) observed substantial differences in signal reductions comparing EMA-treated and non-dye treated killed cells from different species. Using the amplification of a 16S rRNA PCR product of 340 bp as an example, differences in Ct values were reported to be 8, 15.3, >31.4, 7.8, and 11.9 for heat-killed *E. coli*, *Citrobacter koseri*, *Klebsiella oxytoca*, *S. enteritidis*, and *E. sakazakii*, respectively. As cells in the mentioned study were killed by boiling in a water bath, different susceptibilities of the species to stress are an unlikely explanation. One of the reasons could lie in the fact that copy numbers of ribosomal genes can differ substantially between different bacterial species and strains (Klappenbach et al., 2001). As discussed by Contreras et al. (2011), higher copy numbers increase the probability that some of the gene copies have not undergone dye modification and cleavage, which is equivalent with varying amounts of DNA template molecules that can be PCR amplified. The choice of single-copy sequence targets could therefore not only prove beneficial to obtain more consistent results, but might also contribute to more efficient suppression of signals from membrane-compromised cells. Despite the easy availability of ribosomal primers and the resulting experimental convenience, single copy functional genes might be increasingly considered for v-PCR in cases where amplification of multi-copy genes is not required due to low limits of detection.

Other factors contributing to inter-species variation in qPCR results most likely include differences in cell membrane and cell wall complexity. Some of the lowest signal reductions (approx. 7 cycles) when comparing PMA-qPCR results between live and dead cells were obtained for *M. avium* (Nocker et al., 2006) and *M. paratuberculosis* (Kralik et al., 2010). Mycobacterial species are well known for the complexity of their cell envelope. Apart from affecting the permeability of dyes, differences in the complexity and composition of the cell envelope can be assumed to result in varying degrees of membrane damage inferred by a specific stress factor. In other words, the sensitivity of membrane integrity as a viability criterion might vary between species and adds to different susceptibilities of different species to stress. When exposing *V. anguillarum* and *Flavobacterium psychrophilum* to a heat stress gradient, PMA treatment resulted in signal reduction at a lower temperature for the psychrophilic species than for the more heat-tolerant *V. anguillarum* (Contreras et al., 2011). An additional factor contributing to differences between species could potentially be caused by differences in DNA sequences (Chang et al., 2010). As mentioned earlier, preferential binding to DNA containing G–C pairs has been reported for EMA (Dannelley et al., 1986). Sequence heterogeneity in turn can result in differences in DNA conformation and in the display of intercalation sites in the DNA double helix. It remains to be argued that the sequence difference in genes like the 16S rRNA genes might not be sufficiently high to cause the observed discrepancies. Any sequence-caused differences should moreover be minimized when amplifying longer stretches of DNA.

8. Matrix characteristics

Complex matrices as commonly found in environmental samples, food, or clinical samples can negatively influence the efficiency of EMA/PMA treatment (Kramer et al., 2009). Apart from lowering the effective dye concentration by chemical adsorption, organic and inorganic compounds can interfere with photoactivation (Fittipaldi et al., 2011b). Different factors including turbidity, pH, salt, and potentially a higher number of dead cells (for example after disinfection or other treatment) that have the potential to interfere with vPCR results will be discussed in the following.

8.1. Presence of dead cells

The ratio between live and dead cells can affect the efficiency of the method. As demonstrated by some of the authors of this review, defined proportions of viable and non-viable cells were exposed to PMA, followed by quantification of live *L. pneumophila* by qPCR. Quantification of live cells by PMA-PCR in the presence of high levels of dead cells proved difficult in cases when the concentration of live cells was lower than 10^5 cells ml^{-1} (unpublished data). The result resembles the findings by Pan and Breidt (2007). The linear relationship between Ct and the number of viable cells of *L. monocytogenes* was affected when the ratio of dead cells to live cells exceeded 10^4 and the concentration of live cells was less than 10^3 CFU ml^{-1}. The same critical dead/live ratio of 10^4 was published by LØvdal et al. (2011) when applying PMA-qPCR to *L. innocua* and an even smaller ratio was reported for *S. aureus* by Kantonale Laboratorium (2009) stating that dead bacteria should not exceed live bacteria by a factor of 100 without impacting PMA-qPCR and free DNA in copy numbers greater than 4×10^5 can mask the detection of viable cells (Kantonale Laboratorium, 2009). The latter study suffered like several other studies from a lack of validation on whether the 300 W light source was adequate for PMA activation. In some cases the presence of a high number of dead cells might be overcome by increasing the dye concentration. Chang et al. (2010) found that a higher concentration of EMA and PMA was needed in water samples with a larger number of dead cells to achieve comparable results between plate counting and v-PCR.

For studies that did not find an impact of dead cells on vPCR signals, the ratios between dead and live cells did typically not exceed a factor of 1000. When studying the usefulness of EMA and PMA to distinguish between live and dead cells in a mixed bacterial flora from fish fillets, no interference of dead cells was reported when the dead cells constituted 99.6% of the cell population. Similar results, which show that the Ct values were not influenced by the presence of DNA from heat killed cells, have been reported by Wang and Levin (2006) and Chen and Chang (2010). In both cases the ratio of dead cells to viable cells did not exceed 10^3.

The reason for the influence of dead cells on vPCR signals is currently not clear although it appears plausible that high numbers of dead cells with a high capacity of taking up dye would lower the concentration of available dye molecules per cell. Varma et al. (2009) suggested that the effectiveness of PMA activity may be saturated by increasing cell numbers.

8.2. Salt concentration

As early as 1978, EMA photocrosslinking was found to be influenced by the presence of salts (Bolton and Kearns, 1978). In a

study by Graves et al. (1981) both the noncovalent and covalent interactions of EMA with calf thymus DNA were analyzed at three different NaCl concentrations (0.015, 0.0036, and 0.2 M NaCl). Spectrophotometric and dialysis techniques showed that the increasing ionic strength of the buffer resulted in lower apparent association constants of 1.7×10^6, 8.3×10^5, and 2.1×10^5, respectively. The findings might suggest competition between sodium ions and the dye molecules both being positively charged for binding to the negatively charged DNA. Given that salt concentrations will affect electrostatic interactions, the variable yield of photocrosslinking reaction that EMA shows can also be interpreted in terms of a dominant intercalative mechanism at high concentration and both intercalative and stacking mechanisms at low concentrations (Le Pecq and Paoletti, 1967). The efficiency for photolytic attachment of EMA to DNA was found to depend on the ionic strength at defined nucleotide/drug ratios (Graves et al., 1981). Therefore, it is clear that parameters such as the buffer salt concentration and the relationship between nucleotides and drug should be considered.

Whereas the results of the above studies are based on the interaction of dye with purified DNA, a more recent study by Shi et al. (2011) investigated the effect of different factors (including salt) on v-PCR results using cells exposed to different solutions. The experimental design was based on heat-killed cells that were exposed to different concentrations of NaCl (0.125 to 10%, corresponding to a range from approx. 21 mM to 1.7 M) for 30 min. Cells were either left suspended in the salt solution or were harvested and resuspended in water, followed by the addition of EMA (or not). Comparing Ct values with and without EMA, a slight increase in signal reduction was obtained for the latter case when cells from high salt solutions were resuspended in water. The higher the osmotic shock, the higher is the increase in signal reduction. The effect can be attributed to an osmotic destabilization of the cell membrane allowing more efficient dye uptake. Comparing the lowest and highest salt concentration, the effect was however moderate with a difference in signal reduction of only approx. 1 cycle. The second part of the experiment where EMA was directly added to cells suspended in different salt concentrations was in line with before-mentioned studies. Higher salt concentrations resulted in a lower suppression of signals from dead cells by EMA treatment. Whereas EMA performance was comparable for salt concentrations of up to 2%, NaCl concentrations between 4% and 10% reduced EMA signal suppression by approx. 1 to 1.5 cycles. The authors concluded that the presence of elevated salt concentrations should be avoided when EMA treatment is performed, and washing of cells is advisable. It is therefore likely that halophilic microorganisms will need customized procedures to maximize dye performance and minimize cell death during treatment as a consequence of osmotic alterations (Barth et al., 2012; Fittipaldi et al., 2011b; Shi et al., 2011). Recently Barth et al. (2012) found that concentrations of 5% NaCl and above seemed to limit the suppression caused by PMA to DNA from dead cells. Similar results were obtained from working with *Halobacterium salinarum* dead cells or genomic DNA in the presence of NaCl concentrations of 5, 10, 15, and 20%. Additionally they found that the salt concentration in which the *H. salinarum* culture started to lose viability was below the maximum salt concentration allowed to use PMA, limiting the use of the v-PCR technique for cell viability assessment of halophiles and in hypersaline samples.

8.3. Turbidity

Sample turbidity might affect the efficiency of vPCR by reducing the light intensity required for photoactivation of the dye. Photoactivation is both essential for binding of the intercalated dye to DNA and for hydrolytic destruction of excess dye that has not entered cells. Turbidity might be caused by the presence of organic and inorganic compounds or by high densities of microorganisms themselves. Apart from interfering with photoactivation, turbidity caused by organic matter can also act as a cation exchange site lowering the available concentrations of intercalating dye (Pisz et al., 2007).

A number of studies have been performed providing experimental insight. High turbidity caused by high levels of suspended solids was suspected to be the main reason for a lack in efficiency when applying EMA/PMA-qPCR to bacterial quantification in anaerobic sludge (Wagner et al., 2008). A detailed study by Bae and Wuertz (2009) addressed the feasibility of PMA-qPCR analysis for environmental samples containing different concentrations of solids. As expected, lower solid concentrations did not reduce the efficacy of discrimination from heat-killed cells. Higher solid concentration (TSS = 1000 mg/l), on the other hand, was found to affect the suppression of PCR amplification from heat treated cells and required assay optimization (Bae and Wuertz, 2009). It is important to note that the PMA concentration had a statistically significant effect on both viable and dead cells in the presence of higher solid concentrations (TSS = 100 and 1000 mg/l), although signal reductions were much lower for viable cells (Bae and Wuertz, 2009).

In regard to the turbidity threshold that should not be exceeded a remark by Luo et al. (2010) is helpful. Applying PMA to different environmental samples including anaerobic sludge, sediment, and lake mud, Luo et al. (2010) found that the turbidity has no effect on PMA treatment when the turbidities of the sample were less than 10 Nephelometric Turbidity Units (NTU). Similar results were observed by Gedalanga and Olson (2009), who demonstrated that EMA treatment did not adequately differentiate between live and heat-killed cells in primary clarifier and secondary trickling filter effluents with turbidities higher than 10 NTU resulting in false positive results. A practical solution to reduce the influence of turbidity in the efficiency of v-PCR is the dilution of the samples to 1–2 NTU before treatment (Gedalanga and Olson, 2009). The authors point out that dilution might be 'an excellent way to correct turbidity in samples with high concentrations of target bacteria', whereas in the case of low concentrations the relative excess of dye might result in false-negative results in the case of EMA-sensitive populations.

8.4. pH

Shapiro (2003) pointed out that EB can penetrate intact membranes, especially in acid environments. The observation is not surprising given the fact that acid is a potent membrane-disintegrating agent (Alakomi et al., 2000). The damage inferred by low pH on membranes can be assumed to increase the permeability to the dye. This effect has to be differentiated from the one that pH exerts on binding of EMA to DNA. When adding EMA to heat-killed cells suspended in different solutions in a pH range from 1 to 14, Shi et al. (2011) found an interference of low pH with the efficiency of EMA-qPCR. Whereas no effect of pH on the ability of EMA to suppress signals of dead cells was observed for pH 5 and higher, lower pH values resulted in a increasingly strong drop in signal reduction. The effect was observed both for strong and weak acids. The results suggested that the ability of EMA to suppress dead cell signals was weakened at low pH. At pH 1 the same signal was obtained as in the control without EMA treatment. The effect of acidic pH on EMA was supported by absorption spectra showing different maxima at different pH values. Apart from a potential effect on the dye's ability to bind to DNA by structural changes, another reason for the lower efficiency of EMA to suppress signals of dead cells at low pH could lie in the neutralization of the negatively charged DNA weakening the interaction with the dye. The findings suggest that washing of cells is appropriate in the case that cell suspensions are highly acidic.

The influence of pH on PMA treatment was verified by adding PMA to *H. salinarum* genomic DNA or heat inactivated cells in pH values 4.0 and 10 solutions (Barth et al., 2012). The found results

did not show any effect of the hydrogen ion concentration in the PMA action.

9. Summary

A large part of applied microbiological research and quality control is principally or to some extent related with the question whether cells are alive or dead. Despite the availability of a range of techniques for viability assessment, sample treatment with viability dyes received special attention due to its compatibility with DNA amplification procedures. Amplification is typically an essential requirement when using molecular technology for the detection of pathogens which can be present in very low numbers. The inability to selectively detect live cells has been one of the most important obstacles impeding the application of PCR for rapid detection and quantification of microorganisms of interest in food products and water, food processing facilities, and in environmental and clinical samples. Treatment with viability dyes profits from the fact that it is easy to perform, compatible with existing technology and does not significantly increase the time to results. Quantification of live microorganism has been successfully demonstrated within a detection range of 5 log units.

Both dyes, EMA and PMA, are able to suppress the amplification of DNA from cells with damaged membranes or free DNA. Maximum signal reductions of 4–5 log units were reported in many studies applying viability dyes to pure cultures subjected to a lethal treatment that inflicts membrane damage (Chang et al., 2009; Kantonale Laboratorium, 2009; Nocker et al., 2009; Rudi et al., 2002; Rudi et al., 2005b; Varma et al., 2009). In environmental or more complex samples, on the other hand, signal reduction was typically less and depended on a variety of factors. It is important to keep in mind that the efficiency of v-PCR is dependent on the dye concentration and the analyzed microorganisms among other factors. Regarding the latter mentioned factor, PMA has the advantage of a more homogeneous performance with different species. Furthermore, environmental samples, due to the nature of their matrices, could require higher concentrations of dye. As EMA at high concentrations might enter the intact cells of some species, application to samples with mixed species is more problematic. So, the use of PMA has thus a clear advantage in mixed species samples. It is important to underline that the two dyes differ in charge, molecular weight, and membrane permeation properties. In a nutshell, EMA appears more membrane-permeant and can suppress signals of membrane-compromised cells more efficiently than PMA. The higher permeability however also results in penetration of live cells to the extent of being greatly dependent on the microbial species complicating its use for the treatment of samples with mixed species or samples of unknown microbial composition. PMA, on the other hand, affects live cells to a much lesser extent and appears more uniform when applied to different species. The greater selectivity of the less membrane-permeant dye comes however with the price of a somewhat lower maximum signal reduction in the case of dead cells. Different approaches were chosen to overcome the limitations of the two dyes. For EMA, the most important one was to lower the dye concentration (to avoid false-negative signals resulting from dye uptake by live cells) and for PMA to increase the amplicon length (for increased dead-cell exclusion). As the amplification of longer sequences also allows the use of lower dye concentrations, the two parameters are correlated. In regard to long amplicons, an important step has been made by Soejima et al. (2011a,b) by introducing qPCR conditions allowing for the amplification of longer DNA sequences for more efficient suppression of dead cell signals in experimental situations where a large background of membrane-compromised cells is given. The results could shape future v-qPCR amplification strategies as the amplicon length is an easy-to-adjust experimental parameter. Given the fact that currently the majority of commercial pathogen detection kits are optimized for short amplicons, the future might see the development of specific 'long amplicon v-PCR kits'. A balance has to be found between increased amplicon length for better suppression of dead cell signals, amplification efficiency, and detection sensitivity. When applied to EMA, a complication might consist in the penetration of live cells as enhanced signal reduction by long amplicons is not limited to membrane-compromised cells.

Another interesting research direction pursues the identification of treatment conditions that selectively further destabilize membranes of dead cells that have not accumulated sufficient membrane damage. The bile salt deoxycholate has been found to be effective for such selective destabilization when used in low concentrations harsh enough to increase dye uptake for dead cells and gentle enough not to affect the membrane integrity of live cells (Lee and Levin, 2009b). When applied to *V. vulnificus* subjected to freezing or prolonged storage at cold temperatures, a better correlation between EMA-qPCR and plate counts was obtained after treatment with deoxycholate eliminating the erroneous overestimation of live pathogen numbers by EMA-qPCR. The natural detergent has also been found to be effective in *E. coli* (Yang et al., 2011). This strategy to increase dye uptake by dead cells and to increase v-PCR efficiency appears to be primarily relevant for PMA although it has first been applied in combination with EMA treatment. Although the applicability of deoxycholate is limited to Gram-negative bacteria, the studies are an example of an elegant way to improve assay conditions. Other experimental attempts to improve signal reduction by selective enhanced destabilization of dead cells include osmotic or acid stress (Shi et al., 2011) as well as the use of topoisomerase poisons (Soejima et al., 2008). The latter was shown to be effective for suppression of signals from injured cells, but is not applicable to dead cells in which DNA gyrase and topoisomerase IV are not active and ATP is no longer present (Luttinger, 1955; Tanaka et al., 1991). Also a model by some of the authors of this review suggesting the performance of three independent PCR reactions on identical sample aliquots to minimize false-positive and false-negative results can be seen in this line of investigation (Fittipaldi et al., 2011b). The authors suggested one regular qPCR reaction, one v-qPCR reaction, and one v-qPCR on an aliquot subjected to lethal conditions inflicting membrane damage with the intent to provide more objective data regarding the number of live microbes by comparison and subtraction of results. The approach might help to reduce overestimation of bacterial viability in complex matrices like wastewater or fermentor sludge samples.

As addressed in this review, the efficiency of the v-PCR technique depends on a complex set of parameters which include: dye concentration, the microbial species, cell concentration, the ratio between live and dead cells, the length of the PCR amplicon and potentially the sequence of the targeted DNA, the turbidity, pH and salt concentration of the sample, the incubation temperature, and the light source. All factors and their impact of v-PCR require further examination and discussion in order to improve the method. General recommendations that might be useful for assay optimization include the amplification of longer DNA sequences, the preference for single-copy genes, and the choice of an adequate light source. LED light sources can be considered beneficial to prevent unnecessary heating, to minimize DNA damage by wavelengths other than the one required for dye activation, and to avoid unnecessary optimization of light exposure time and testing of the suitability of the light source. In cases where dye penetration into live cells is problematic, EMA incubation at low temperatures (e.g., 4 °C) might increase the selectivity for dead cells. The incubation temperature for PMA should probably not be lower than 20 °C in order to not decrease cell membrane permeability. When handling samples with high acidity ($pH \leq 4$), high salt concentrations ($\geq 4\%$), or high turbidity (≥ 10 NTU), pH adjustment, harvesting and resuspension of cells and dilution of samples are advisable, respectively. As the dyes have a

limited capacity to exclude dead cell signals, spiking of samples with increasing defined numbers of dead microorganisms might help to identify the dead cell exclusion limit and ultimately improve the accuracy of live cell prediction. Alternatively, standard curves obtained from spiking with known numbers of live cells might be considered. And last but not least, v-qPCR, like regular qPCR reactions, can greatly profit from the inclusion of internal amplification controls that indicate interference of excess dye or other experimental factors with amplification efficiency.

Although many studies found consistencies between v-qPCR results and plate counts (Andorrà et al., 2010; Vesper et al., 2008), a discrepancy in live cell numbers might be caused by the presence of sublethally injured cells as they tend to occur when applying mild disinfection treatment or during stress exposure. Two scenarios are conceivable. In the first cell injury interferes with colony formation without affecting membrane integrity, in the second cells still grow, but the stress temporarily compromises membrane-integrity and allows dye uptake. Whereas the first case results in higher live cell numbers by v-qPCR (as observed after chlorine treatment, Nocker et al., 2007b), the second results in higher numbers by plate counts (Kantonale Laboratorium, 2009; Shi et al., 2011). A short incubation of samples in recovery medium could potentially minimize the discrepancy. Examples for recovery procedure have been successfully described for yeast (Andorrà et al., 2010) and *E. coli* (Shi et al., 2011). Incubation times were chosen long enough to allow damage repair, but short enough to prevent replication of live cells.

The principle of live–dead distinction in v-PCR is based on membrane integrity. Despite the common use of this conservative indicator for viability, it is important to understand its limitations. EMA or PMA like other membrane integrity stains do not measure “life” or “death”, but assess a particular location-specific damage to cells (Hammes et al., 2011). Cell death can therefore only be diagnosed by v-PCR if it is reflected in membrane damage. The diagnostic method cannot be applied to cells subjected to biocidal treatments that do not or only insufficiently inflict membrane damage in the relevant dose range with UV being a classical example (Nocker et al., 2007b; Lee and Levin, 2009a). On the other hand, the treatments that affect membrane integrity vary in the degree of damage that is inflicted before cell death occurs. A study describing the effect of different treatments on selected viability parameters and culturability has recently been performed for *L. monocytogenes* exposed to elevated temperature, pH, high salt concentrations, a detergent disinfectant and an oxidative disinfectant (Nocker et al., 2011). An overall correlation of 0.82 was found between membrane integrity (as assessed by the BacLight Live/Dead staining kit) and culturability on plate. Exposure to the detergent disinfectant was the treatment providing the best correlation between membrane integrity and culturability, which is not surprising given the fact that the detergent component of the disinfectant directly targets the cell membrane. For other treatments, loss of redox and esterase activity tended to better correlate with loss of culturability. For these scenarios, cell death might already have occurred before the membrane integrity suffered detectable damage. These results supported an earlier statement that loss of membrane integrity might be a relatively ‘conservative’ viability criterion (Nocker et al., 2007b). On the other hand, drying might inflict temporary membrane damage without necessarily impacting culturability as observed for the desiccation-tolerant *S. aureus* (Kantonale Laboratorium, 2009). It is highly unlikely that a ‘golden bullet’ viability assessment method exists due to the heterogeneous nature of microbial life (Hammes et al., 2011). Multiple cellular criteria might have to be assessed before definite conclusions on the cell's viability are possible. Membrane integrity can be considered as a widely used and accepted viability criterion. In the future a combination of PCR with viability parameters other than membrane integrity might be achieved (Nocker and Camper, 2009). The presence of an electrochemical gradient and/or cellular metabolism was recently discussed as additional components that could be added to the definition of viability (Trevors, 2012). However, already in its current state, despite the need for a more efficient exclusion of dead cells, sample treatment with viability dyes with subsequent amplification of DNA can be seen as highly valuable for a wide range of applications.

Disclosure statement

We would like to declare author relationships that can be viewed as presenting a potential conflict of interest. Nowadays, Francesc Codony and Mariana Fittipaldi are working for GenIUL S.L. and Andreas Nocker is related with the PMA patent.

References

Agusti, G., Codony, F., Fittipaldi, M., Adrados, B., Morató, J., 2010. Viability determination of *Helicobacter pylori* using propidium monoazide quantitative PCR. Helicobacter 15, 473–476.

Alakomi, H.L., SkyttÄ, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., Helander, I.M., 2000. Lactic acid permeabilizes Gram-negative bacteria by disrupting the outer membrane. Appl. Environ. Microbiol. 66, 2001–2005.

Alifano, P., Bruni, C.B., Carlomagno, M.S., 1994. Control of messenger-RNA processing and decay in prokaryotes. Genetica 94, 157–172.

Andorrà, I., Esteve-Zarzoso, B., Guillamón, J.M., Mas, A., 2010. Determination of viable wine yeast using DNA binding dyes and quantitative PCR. Int. J. Food Microbiol. 144, 257–262.

Bae, S., Wuertz, S., 2009. Discrimination of viable and dead fecal *Bacteroidales* bacteria by quantitative PCR with propidium monoazide. Appl. Environ. Microbiol. 75, 2940–2944.

Banihashemi, A., Van Dyke, M.I., Huck, P.M., 2012. Long-amplicon propidium monoazide-PCR enumeration assay to detect viable *Campylobacter* and *Salmonella*. J. Appl. Microbiol. http://dx.doi.org/10.1111/j.1365-2672.2012.05382.x.

Barni, S., Polver, P.D., Gerzeli, G., Nano, R., 1981. Propidium iodide as a probe for the study of chromatin thermal denaturation in situ. Histochem. J. 13, 781–791.

Barth Jr., V.C., Cattani, F., Ferreira, C.A.S., de Oliveira, S.D., 2012. Sodium chloride affects propidium monoazide action to distinguish viable cells. Anal. Biochem. 428, 108–110.

Blake, A., Peacocke, A.R., 1968. Perspectives report: the interaction of aminoacridines with nucleic acids. Biopolymers 6, 1225–1253.

Bleve, G., Rizzotti, L., Dellaglio, F., Torriani, S., 2003. Development of reverse transcription (RT)-PCR and real-time RT-PCR assays for rapid detection and quantification of viable yeasts and molds contaminating yogurts and pasteurized food products. Appl. Environ. Microbiol. 69, 4116–4122.

Bolton, P.H., Kearns, D.R., 1978. Spectroscopic properties of ethidium monoazide: a fluorescent photoaffinity label for nucleic acids. Nucleic Acids Res. 5, 4891–4903.

Brescia, C.C., Griffin, S.M., Ware, M.W., Varughese, E.A., Egorov, A.I., Villegas, E.N., 2009. *Cryptosporidium* propidium monoazide-PCR, a molecular biology-based technique for genotyping of viable *Cryptosporidium* oocysts. Appl. Environ. Microbiol. 75, 6856–6863.

Cawthorn, D.M., Witthuhn, R.C., 2008. Selective PCR detection of viable *Enterobacter sakazakii* cells utilizing propidium monoazide or ethidium bromide monoazide. Appl. Environ. Microbiol. 105, 1178–1185.

Chang, B., Sugiyama, K., Taguri, T., Amemura-Maekawa, J., Kura, F., Watanabe, H., 2009. Specific detection of viable *Legionella* cells by combined use of photoactivated ethidium monoazide and PCR/real-time PCR. Appl. Environ. Microbiol. 75, 147–153.

Chang, B., Taguri, T., Sugiyama, K., Amemura-Maekawa, J., Kura, F., Watanabe, H., 2010. Comparison of ethidium monoazide and propidium monoazide for the selective detection of viable *Legionella* cells. Jpn. J. Infect. Dis. 63, 119–123.

Chen, N.T., Chang, C.W., 2010. Rapid quantification of viable legionellae in water and biofilm using ethidium monoazide coupled with real-time quantitative PCR. J. Appl. Microbiol. 109, 623–634.

Chen, S., Wang, F., Beaulieu, J.C., Stein, R.E., Ge, B., 2011. Rapid detection of viable salmonellae in produce by coupling propidium monoazide with loop-mediated isothermal amplification. Appl. Environ. Microbiol. 77, 4008–4016.

Contreras, P.J., Urrutia, H., Sossa, K., Nocker, A., 2011. Effect of PCR amplicon length on suppressing signals from membrane-compromised cells by propidium monoazide treatment. J. Microbiol. Methods 87, 89–95.

Dannelley, J.M., Boyce, L., Gaubatz, J.W., 1986. Efficiency of photoaffinity labeling DNA homopolymers and copolymers with ethidium monoazide. Photochem. Photobiol. 43, 7–11.

Delgado Viscogliosi, P., Solignac, L., Delattre, J.M., 2009. Viability PCR, a culture independent method for rapid and selective quantification of viable *Legionella pneumophila* cells in environmental water samples. Appl. Environ. Microbiol. 75, 3502–3512.

DeTraglia, M.C., Brand, J.S., Tometsko, A.M., 1978. Characterization of azidobenzamidines as photoaffinity labels for trypsin. J. Biol. Chem. 253, 1846–1852.

Douglas Inglis, D., McAllister, T.M., Larney, F.J., Topp, E., 2010. Prolonged survival of *Campylobacter* species in bovine manure compost. Appl. Environ. Microbiol. 76, 1110–1119.

Elizaquivel, P., Sanchez, G., Selma, M.V., Aznar, R., 2012. Application of propidium monoazide-qPCR to evaluate the ultrasonic inactivation of *Escherichia coli* O157:H7 in fresh-cut vegetable wash water. Food Microbiol. 30, 316–320.

Fittipaldi, M., Rodriguez, N.J., Codony, F., Adrados, B., Peñuela, G., Morató, J., 2010. Discrimination of infectious bacteriophage T4 virus by propidium monoazide real-time PCR. J. Virol. Methods 168, 228–232.
Fittipaldi, M., Pino Rodriguez, N.J., Adrados, B., Agustí, G., Peñuela, G., Morató, J., 2011a. Discrimination of viable *Acanthamoeba castellani* trophozoites and cysts by propidium monoazide real-time polymerase chain reaction. J. Eukaryot. Microbiol. 58, 359–364.
Fittipaldi, M., Codony, F., Adrados, B., Camper, A.K., Morato, J., 2011b. Viable real-time PCR in environmental samples: can all data be interpreted directly? Microb. Ecol. 61, 7–12.
Flekna, G., Stefanic, P., Wagner, M., Smulders, F.J., Mozina, S.S., Hein, I., 2007. Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. Res. Microbiol. 158, 405–412.
Garbett, N.C., Hammond, N.B., Graves, D.E., 2004. Influence of the amino substituents in the interaction of ethidium bromide with DNA. Biophys. J. 87, 3974–3981.
Garland, F., Graves, D.E., Yielding, L.W., Cheung, H.C., 1980. Comparative studies of the binding of ethidium bromide and its photoreactive analogues to nucleic acids by fluorescence and rapid kinetics. Biochemistry 19, 3221–3226.
Gedalanga, P.B., Olson, B.H., 2009. Development of a quantitative PCR method to differentiate between viable and nonviable bacteria in environmental water samples. Appl. Microbiol. Biotechnol. 82, 587–596.
Graiver, D.A., Saunders, S.E., Topliff, C.L., Kelling, C.L., Bartelt-Hunt, S.L., 2010. Ethidium monoazide does not inhibit RT-PCR amplification of nonviable avian influenza RNA. J. Virol. Methods 164, 51–54.
Graves, D.E., Watkins, C.L., Yielding, L.W., 1981. Ethidium bromide and its photoreactive analogues: spectroscopic analysis of deoxyribonucleic acid binding properties. Biochemistry 20, 1887–1892.
Greulich, K.O., 2004. Single molecule techniques for biomedicine and pharmacology. Curr. Pharm. Biotechnol. 5, 243–259.
Hammes, F., Berney, M., Egli, T., 2011. Cultivation-independent assessment of bacterial viability. Adv. Biochem. Eng. Biotechnol. 124, 123–150.
Hardwick, J.M., von Sprecken, R.S., Yielding, K.L., Yielding, L.W., 1984. Ethidium binding sites on plasmid DNA determined by photoaffinity labeling. J. Biol. Chem. 259, 11090–11097.
Jernaes, M.W., Steen, H.B., 1994. Staining of *Escherichia coli* for flow cytometry: influx and efflux of ethidium bromide. Cytometry 17, 302–309.
Kantonale Laboratorium, 2009. Development of a method to distinguish between DNA from dead or living microorganisms. EFBS-Project Report 04.1240.PZ/H355-0649. http://www.efbs.admin.ch/fileadmin/efbsdateien/dokumentation/Publikationen/Report_PMA_Project_final.pdf.
Klappenbach, J.A., Saxman, P.R., Cole, J.R., Schmidt, T.M., 2001. rrndb: the ribosomal RNA operon copy number database. Nucl. Acids Res. 29, 181–184.
Kobayashi, H., Oethinger, M., Tuohy, M.J., Hall, G.S., Bauer, T.W., 2009. Unsuitable distinction between viable and dead *Staphylococcus aureus* and *Staphylococcus epidermis* by ethidium bromide monoazide. Lett. Appl. Microbiol. 48, 633–638.
Kralik, P., Nocker, A., Pavlik, I., 2010. *Mycobacterium avium* subsp. *paratuberculosis* viability determination using F57 quantitative PCR in combination with propidium monoazide treatment. Int. J. Food Microbiol. 141 (Suppl. 1), S80–S86.
Kramer, M., Obermajer, N., Bogovic Matijasic, B., Rogelj, I., Kmetec, V., 2009. Quantification of live and dead probiotic bacteria in lyophilised product by real time PCR and flow cytometry. Appl. Microbiol. Biotechnol. 84, 1137–1147.
Laugaa, P., Delbarre, A., Le Pecq, J.B., Roques, B.P., 1983. Comparative binding of ethidium and three azido analogs to dinucleotides: affinity and intercalation geometry. Eur. J. Biochem. 134, 163–173.
Le Pecq, J.B., Paoletti, C., 1967. A fluorescent complex between ethidium bromide and nucleic acids: physical–chemical characterization. J. Mol. Biol. 27, 87–106.
Lee, J.L., Levin, R.E., 2009a. A comparative study of the ability of EMA and PMA to distinguish viable from heat killed bacterial flora from fish fillets. J. Microbiol. Methods 76, 93–96.
Lee, J.L., Levin, R.E., 2009b. Discrimination of viable and dead *Vibrio vulnificus* after refrigerated and frozen storage using EMA, sodium deoxycholate and real-time PCR. J. Microbiol. Methods 79, 184–188.
Li, B., Chen, J.Q., 2012. Real-time PCR methodology for selective detection of viable *Escherichia coli* O157:H7 by targeting Z3276 as a genetic marker. Appl. Environ. Microbiol. http://dx.doi.org/10.1128/AEM.00794-12.
Liang, N., Dong, J., Luo, L., Li, Y., 2011. Detection of viable *Salmonella* in lettuce by propidium monoazide real-time PCR. J. Food Sci. 76 (4), M234–M237.
Loozen, G., Boon, N., Pauwels, M., Quirynen, M., Teughels, W., 2011. Live/dead real-time polymerase chain reaction to assess new therapies against dental plaque-related pathologies. Mol. Oral Microbiol. 26, 253–261.
Løvdal, T., Hovda, M.B., Björkblom, B., Møller, S.G., 2011. Propidium monoazide combined with real-time quantitative PCR underestimates heat-killed *Listeria innocua*. J. Microbiol. Methods 85, 164–169.
Lu, Y., Yang, W., Shi, L., Li, L., Alam, M.J., Guo, S., et al., 2009. Specific detection of viable *Salmonella* cells by an ethidium-monoazide-loop mediated isothermal amplification (EMA-LAMP) method. J. Health Sci. 55, 820–824.
Luo, J.F., Lin, W.T., Guo, Y., 2010. Method to detect only viable cells in microbial ecology. Appl. Microbiol. Biotechnol. 86, 377–384.
Luttinger, A., 1955. The twisted "life" of DNA in the cell: bacterial topoisomerases. Mol. Microbiol. 15, 601–606.
Marouani-Gradi, N., Firmesse, O., Chassaing, D., Sandris-Nielsen, D., Arneborg, N., Carpentier, B., 2010. Potential of *Escherichia coli* O157:H7 to persist and form viable but non-culturable cells on a food-contact surface subjected to cycles of soiling and chemical treatment. Int. J. Food Microbiol. 15, 96–103.
Martin, B., Raurich, S., Garriga, M., Aymerich, T., 2012. Effect of amplicon length in propidium monoazide quantitative PCR for the enumeration of viable cells of *Salmonella* in cooked ham. Food Anal. Methods http://dx.doi.org/10.1007/s12161-012-9460-0.
Marx, G., Zhou, H., Graves, D.E., Osheroff, N., 1997. Covalent attachment of ethidium to DNA results in enhanced topoisomerase II mediated DNA cleavage. Biochemistry 36, 15884–15891.
Meng, X.C., Pang, R., Wang, C., Wang, L.Q., 2010. Rapid and direct quantitative detection of viable bifidobacteria in probiotic yogurt by combination of ethidium monoazide and real-time PCR using a molecular beacon approach. J. Dairy Res. 77, 498–504.
Minami, J., Yoshida, K., Soejima, T., Yaeshima, T., Iwatsuki, K., 2010. New approach to use ethidium monoazide as an analytical tool. J. Appl. Microbiol. 109, 900–909.
Miotto, P., Bigoni, S., Migliori, G.B., Matteelli, A., Cirillo, D.M., 2012. Early tuberculosis treatment monitoring by Xpert MTB/RIF. Eur. Respir. J. 39, 1269–1271.
Morin, N.J., Gong, Z., Li, X.F., 2004. Reverse transcription-multiplex PCR assay for simultaneous detection of *Escherichia coli* O157: H7, *Vibrio cholerae* O1, and *Salmonella typhi*. Clin. Chem. 50, 2037–2044.
Nam, S., Kwon, S., Kim, M.J., Chae, J.C., Jae Maeng, P., Park, J.G., et al., 2011. Selective detection of viable *Helicobacter pylori* using ethidium monoazide or propidium monoazide in combination with real-time polymerase chain reaction. Microbiol. Immunol. 55, 841–846.
Nebe-von-Caron, G., Stephens, P.J., Hewitt, C.J., Powell, J.R., Badley, R.A., 2000. Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. J. Microbiol. Methods 42, 97–114.
Nikaido, H., 2001. Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. Semin. Cell Dev. Biol. 12, 215–223.
Nocker, A., Camper, A.K., 2006. Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. Appl. Environ. Microbiol. 72, 1997–2004.
Nocker, A., Camper, A.K., 2009. Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. FEMS Microbiol. Lett. 291, 137–142.
Nocker, A., Cheung, C.Y., Camper, A.K., 2006. Comparison of propidium monoazide and ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J. Microbiol. Methods 67, 310–320.
Nocker, A., Sossa-Fernandez, P., Burr, M.D., Camper, A.K., 2007a. Use of propidium monoazide for live/dead distinction in microbial ecology. Appl. Environ. Microbiol. 73, 5111–5117.
Nocker, A., Sossa, K.E., Camper, A.K., 2007b. Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. J. Microbiol. Methods 70, 252–260.
Nocker, A., Mazza, A., Masson, L., Camper, A.K., Brousseau, R., 2009. Selective detection of live bacteria combining propidium monoazide sample treatment with microarray technology. J. Microbiol. Methods 76, 253–261.
Nocker, A., Richter, T., Schuren, F., Montijn, R., Kort, R., 2010. Live/dead discrimination in 454 pyrosequencing-based bacterial community analysis of environmental water samples. Int. Microbiol. 13, 59–65.
Nocker, A., Caspers, M., Esveld-Amanatidou, A., van der Vossen, J., Schuren, F., Montijn, R., et al., 2011. Multiparameter viability assay for stress profiling applied to the food pathogen *Listeria monocytogenes* F2365. Appl. Environ. Microbiol. 77, 6433–6440.
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 810, 812–813.
Pan, Y., Breidt, F., 2007. Enumeration of viable *Listeria monocytogenes* cells by real time PCR with propidium monoazide and ethidium monoazide in the presence of dead cells. Appl. Environ. Microbiol. 73, 8028–8031.
Pflaum, M., Boiteux, S., Epe, B., 1994. Visible light generates oxidative DNA base modifications in high excess of strand breaks in mammalian cells. Carcinogenesis 15, 297–300.
Pisz, J.M., Lawrence, J.R., Schafer, A.N., Siciliano, S.D., 2007. Differentiation of genes extracted from nonviable versus viable micro-organisms in environmental samples using ethidium monoazide bromide. J. Microbiol. Methods 71, 312–318.
Qin, T., Tian, Z., Ren, H., Hu, G., Zhou, H., Lu, J., et al., 2012. Application of EMA-qPCR as a complementary tool for the detection and monitoring of *Legionella* in different water systems. World J. Microbiol. Biotechnol. 28, 1881–1890.
Rawsthorne, H., Dock, C.N., Jaykus, L.A., 2009. PCR-based method using propidium monoazide to distinguish viable from non-viable *Bacillus subtilis* spores. Appl. Environ. Microbiol. 75, 2936–2939.
Rodriguez, L., Ramos, J., Couto, I., Amaral, L., Viveiros, M., 2011. Ethidium bromide transport across *Mycobacterium smegmatis* cell-wall: correlation with antibiotic resistance. BMC Microbiol. 11, 2–10.
Rogers, G.B., Stressmann, F.A., Koller, G., Daniels, T., Carroll, M.P., Bruce, K.D., 2008. Assessing the diagnostic importance of nonviable bacterial cells in respiratory infections. Diagn. Microbiol. Infect. Dis. 62, 133–141.
Rudi, K., Nogva, H.K., Moen, B., Nissen, H., Bredholt, S., Moretro, T., et al., 2002. Development and application of new nucleic acid-based technologies for microbial community analyses in foods. Int. J. Food Microbiol. 78, 171–180.
Rudi, K., Moen, B., Drømtorp, S.M., Holck, L., 2005a. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl. Environ. Microbiol. 71, 1018–1024.
Rudi, K., Naterstad, K., Dromtorp, S.M., Holo, H., 2005b. Detection of viable and dead *Listeria monocytogenes* on gouda-like cheeses by real-time PCR. Lett. Appl. Microbiol. 40, 301–306.
Rueckert, A., Ronimus, R.S., Morgan, H.W., 2005. Rapid differentiation and enumeration of the total, viable vegetative cell and spore content of thermophilic bacilli in milk powders with reference to *Anoxybacillus flavithermus*. J. Appl. Microbiol. 99, 1246–1255.
Sanchez, G., Elizaquivel, P., Aznar, R., 2012. Discrimination of infectious hepatitis A viruses by propidium monoazide real-time RT-PCR. Food Environ. Virol. 4, 21–25.

Schumacher, A., Trittler, R., Bohnert, J.A., Kümmerer, K., Pagès, J.M., Kern, W.V., 2007. Intracellular accumulation of linezolid in *Escherichia coli*, *Citrobacter freundii* and *Enterobacter aerogenes*: role of enhanced efflux pump activity and inactivation. J. Antimicrob. Chemother. 59, 1261–1264.

Shapiro, H.M., 2003. Parameters and Probes, 4th edition. John Wiley & Sons, Inc., Hoboken, New Jersey, p. 369.

Sheridan, G.E.C., Masters, C.I., Shallcross, J.A., Mackey, B.M., 1998. Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells. Appl. Environ. Microbiol. 64, 1313–1318.

Shi, H., Xu, W., Luo, Y., Chen, L., Liang, Z., Zhou, X., et al., 2011. The effect of various environmental factors on the ethidium monoazide and quantitative PCR method to detect viable bacteria. J. Appl. Microbiol. 111, 1194–1204.

Shi, H., Xu, W., Trinh, Q., Luo, Y., Liang, Z., Li, Y., et al., 2012. Establishment of a viable cell detection system for microorganisms in wine based on ethidium monoazide and quantitative PCR. Food Control 27, 81–86.

Soejima, T., Iida, K., Qin, T., Taniai, H., Seki, M., Takade, A., et al., 2007. Photoactivated ethidium monoazide directly cleaves bacterial DNA and is applied to PCR for discrimination of live and dead bacteria. Microbiol. Immunol. 51, 763–775.

Soejima, T., Iida, K., Qin, T., Taniai, H., Seki, M., Yoshida, S., 2008. Method to detect only live bacteria during PCR amplification. J. Clin. Microbiol. 46, 2305–2313.

Soejima, T., Schlitt-Dittrich, F., Yoshida, S., 2011a. Polymerase chain reaction length-dependent ethidium monoazide suppression power for heat-killed *Enterobacteriaceae*. Anal. Biochem. http://dx.doi.org/10.1016/j.ab.2011.06.027.

Soejima, T., Schlitt-Dittrich, F., Yoshida, S., 2011b. Rapid detection of viable bacteria by nested polymerase chain reaction via long DNA amplification after ethidium monoazide treatment. Anal. Biochem. 418, 37–43.

Tanaka, M., Sato, K., Kimura, Y., Hayakawa, I., Osada, Y., Nishino, T., 1991. Inhibition by quinolones of DNA gyrase from *Staphylococcus aureus*. Antimicrob. Agents Chemother. 35, 1489–1491.

Taskin, B., Gozen, A.G., Duran, M., 2011. Selective quantification of viable Escherichia coli bacteria in biosolids by quantitative PCR with propidium monoazide modification. Appl. Environ. Microbiol. 77, 4329–4335.

Trevors, J.T., 2012. Can dead bacterial cells be defined and are genes expressed after cell death? J. Microbiol. Methods 90, 25–28.

Vaitilingom, M., Gendre, F., Brignon, P., 2006. Direct detection of viable bacteria, molds, and yeasts by reverse transcriptase PCR in contaminated milk samples after heat treatment. Appl. Environ. Microbiol. 72, 1997–2004.

Van de Vossenberg, J.L., Ubbink-kok, T., Elferink, M.G., Driessen, A.J., Konings, W.N., 1995. Ion permeability of the cytoplasmatic membrane limits the maximum growth temperature of bacteria and archaea. Mol. Microbiol. 18, 925–932.

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 Res. 43, 4790–4801.

Vesper, S., McKinstry, C., Hartmann, C., Neace, M., Yoder, S., Vesper, A., 2008. Quantifying fungal viability in air and water samples using quantitative PCR after treatment with propidium monoazide (PMA). J. Microbiol. Methods 72, 180–184.

Wagner, A.O., Malin, C., Knapp, B.A., Illmer, P., 2008. Removal of free extracellular DNA from environmental samples by ethidium monoazide and propidium monoazide. Appl. Environ. Microbiol. 74, 2537–2539.

Wang, S., Levin, R.E., 2006. Discrimination of viable *Vibrio vulnificus* cells from dead cells in real-time PCR. J. Microbiol. Methods 64, 1–8.

Wang, L., Li, Y., Musthapha, A., 2009. Detection of viable *Escherichia coli* O157:H7 by ethidium monoazide real time PCR. J. Appl. Microbiol. 107, 1719–1728.

Wang, L., Zhong, Q., Li, Y., 2012. Ethidium monoazide-loop mediated isothermal amplification for rapid detection of *Vibrio parahaemolyticus* in viable but non-culturable state. Energy Procedia 17, 1858–1863.

Waring, M.J., 1965. Complex formation between ethidium bromide and nucleic acids. J. Mol. Biol. 13, 269–282.

White, W.E., Yielding, K.L., 1977. Photoaffinity labels for nucleic acids. Methods Enzymol. 46, 644–649.

Wilson, W.D., Jones, R.L., Zon, G., Banville, D.J., Marzilli, L.G., 1986. Specificity in DNA interactions: an NMR investigation of the interaction of propidium with oligodeoxyribonucleotides containing normal and G·T base pairs. Biopolymers 25, 1997–2015.

Yañez, M.A., Nocker, A., Soria-Soria, E., Múrtula, R., Martinez, L., Catalán, V., 2011. Quantification of viable *Legionella pneumophila* cells using propidium monoazide combined with quantitative PCR. J. Microbiol. Methods 85, 124–130.

Yang, X., Badoni, M., Gill, C.O., 2011. Use of propidium monoazide and quantitative PCR for differentiation of viable *Escherichia coli* from *E. coli* killed by mild or pasteurizing heat treatments. Food Microbiol. 28, 1478–1482.

Yielding, L.W., Firth III, W.J., 1980. Structure–function characterization for ethidium photoaffinity labels as mutagens in *Salmonella*. Mutat. Res. 71, 161–168.

Yielding, L.W., Yielding, K.L., Donoghue, J.E., 1984. Ethidium binding to deoxyribonucleic acid: spectrophotometric analysis of analogs with amino, azido, and hydrogen substituents. Biopolymers 23, 83–110.