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Quantification of Viable *Legionella pneumophila* Cells Using Propidium

Monoazide Combined with quantitative PCR

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Abstract

One of the greatest challenges of implementing fast molecular detection methods as part of *Legionella* surveillance systems is to limit detection to live cells. In this work, a protocol for sample treatment with propidium monoazide (PMA) in combination with quantitative PCR (qPCR) has been optimized and validated for *L. pneumophila* as an alternative of the currently used time-consuming culture method. Results from PMA-qPCR were compared with culture isolation and traditional qPCR. Under the conditions used, sample treatment with 50 μ M PMA followed by 5 min of light exposure were assumed optimal resulting in an average reduction of 4.45 log units of the qPCR signal from heat-killed cells. When applied to environmental samples (including water from cooling water towers, hospitals, spas, hot water systems in hotels, and tap water), different degrees of correlations between the three methods were obtained which might be explained by different matrix properties, but also varying degrees of non-culturable cells. It was furthermore shown that PMA displayed substantially lower cytotoxicity with *Legionella* than the alternative dye ethidium monoazide (EMA) when exposing live cells to the dye followed by plate counting. This result confirmed findings with other species that PMA is less membrane-permeant and more selective for intact cells. In conclusion, PMA-qPCR is a promising technique for limiting detection to intact cells and makes *Legionella* surveillance data substantially more relevant in comparison with qPCR alone. For future research it would be desirable to increase the method's capacity to exclude signals from dead cells in difficult matrices or samples containing high numbers of dead cells.

KEY WORDS: *Legionella pneumophila*, PMA, qPCR, viable cells.

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1. Introduction

Legionella pneumophila is one of the main causative agents of severe atypical pneumonias, particularly among people with impaired immune systems. Present in soils and natural aquatic environments, *Legionella* can persist as free-living microorganisms, as part of biofilms, or as intracellular parasites of amoebae and ciliates (Brand and Hacker, 1996; Steinert et al., 2002). *L. pneumophila* has found an appropriate ecological niche in several man-made aquatic environments such as potable water systems, cooling towers, evaporative condensers, and wastewater systems (Colbourne et al., 1988). Legionnaires' disease (LD) is caused mainly by inhalation of aerosols generated from soil or aquatic environments contaminated with *Legionella* (Pascual et al., 2001). Outbreaks of Legionellosis occur throughout the world affecting public health as well as various industrial, tourist, and social activities (Sabria and Campins, 2003). For this reason, surveillance systems have been implemented in many countries. These programs have reduced the risk to a tolerable minimum and for example reduced the frequency with which nosocomial *L. pneumophila* was isolated from hospital patients with pneumonia from 16.6% to 0.1% in a six-year period in Germany (Junge-Mathys and Mathys, 1994).

The assessment of *L. pneumophila* in water samples is typically performed by culture isolation on selective media (European Guidelines, 2005). However, all culture-based methods applied to the analysis of *Legionella* require long incubation times due to the slow growth rate of the bacterium and do not permit the detection of viable but non-culturable bacteria (VBNC) that may represent a public health hazard. Moreover, it is difficult to isolate *Legionella* in samples containing high levels of other microorganisms.

To overcome these limitations, nucleic acid amplification techniques and mainly PCR methodologies have been described as useful tools for the detection of *Legionella* ssp. and specifically for *L. pneumophila* in clinical and environmental samples (Miyamoto et al., 1997; Yáñez et al., 2007). The main advantages of these PCR methodologies are high specificity, sensitivity, rapidity, low limit of detection, and the possibility of quantifying the microorganism using quantitative PCR (qPCR). The application of qPCR for direct detection and quantification of *Legionella* in environmental and clinical samples is rapidly increasing (Bustin et al., 2009) with a large number of different protocols available (Ballard et al., 2000; Hayden et al., 2001; Herpers et al., 2003; Rantakokko-Jalava et al., 2001; Yáñez et al., 2005). Nevertheless, although the application of all these methods has greatly improved the environmental and clinical diagnostics of *Legionella*, two major limitations are known, including the potential presence of PCR inhibitors that can result in false-negative results, and the inability of PCR to differentiate between live and dead cells in case that DNA serves as a molecular target. Whereas the first drawback can be relatively easily solved by including internal positive controls (IPCs) in the PCR reaction, the latter poses a severe challenge as DNA can persist for long periods after cell death (Josephson et al., 1993). This is particularly relevant when disinfection is performed and killing efficiency is monitored directly after the disinfection procedure. The time between cell death and DNA detection is normally far shorter than the time required for DNA degradation. The inability of live/dead differentiation may lead to an overestimation of the actual sanitary risk, and is therefore a serious limitation for implementing DNA-based diagnostics in routine applications.

The majority of current molecular methods for viability assessment propose the use of mRNA and rRNA. However, the longer half-life of rRNA species and their variable retention following a variety of bacterial stress treatments make rRNA a less suitable indicator of viability than mRNA (Dreier et al., 2004). The most commonly used amplification techniques for detecting mRNA are reverse transcriptase PCR (RT-PCR), nucleic acid sequence-based amplification (NASBA) (Kievits et al., 1991), and reverse transcriptase-strand displacement amplification (RT-SDA) (Walker et al., 1992). Nevertheless, working with mRNA is far from trivial. Problems associated with the use of mRNA are mostly related to the difficulty of quantification since the number of target mRNA molecules does not reflect the number of cells and greatly depends on the metabolic activity of the cells. Additionally, some mRNA molecules are not transcribed in cells in the VBNC state (Yaron et al., 2002).

A promising approach to overcome the lack of viability information in DNA-based detection methods was described by Nogva et al. 2003 introducing the viability dye ethidium monoazide (EMA). The combination of sample treatment with EMA with subsequent qPCR analysis makes use of the speed and sensitivity of molecular detection while at the same time providing viability information. The elegant method is based on the addition of EMA to the sample prior analysis. Samples are incubated for some minutes allowing the dye to penetrate membrane-compromised cells and to intercalate into the DNA of these cells. Subsequent light exposure results in fragmentation of the such modified DNA (Soejima et al., 2007) which reduces the amplifiability of the DNA template. Despite the effectiveness of EMA to reduce PCR signals from killed cells, a disadvantage consists in the fact that the compound can also enter live cells with intact

membranes with species-dependent differences (Nocker et al., 2006). This phenomenon has been described for various microorganisms including *Anoxybacillus* (Rueckert et al., 2005), *Campylobacter jejuni*, *Escherichia coli* 0157:H7, *Listeria monocytogenes*, *Micrococcus luteus*, and *Mycobacterium avium* (Flekna et al., 2007; Nocker and Camper, 2006), *Staphylococcus aureus* and *Staphylococcus epidermis* (Kobayashi et al., 2009). Therefore, propidium monoazide (PMA) combined with qPCR (PMA-qPCR) has been proposed as an alternative method (Nocker et al., 2006). The higher selectivity for live cells was hypothesized to be due to the higher charge of PMA (two positive charges) compared to EMA (one positive charge) making it more difficult for PMA to penetrate intact cell membranes. The PMA concept has in the meantime been successfully applied to a wide range of bacteria including *Acinetobacter* sp., *Aeromonas culicula*, *Aeromonas salmonicida*, *Alcaligenes faecalis*, Bacteroidales, *Burkholderia cepacia*, *Enterobacter aerogenes*, *Enterobacter sakazakii*, *Escherichia coli* (includ. *Escherichia coli* O157:H7), *Klebsiella* sp., *Listeria monocytogenes*, *Mycobacterium avium* complex subsp. *avium*, *Mycobacterium avium* subsp. *paratuberculosis*, *Nitrosomonas europaea*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium and *Staphylococcus aureus* (Bae and Wuertz, 2009; Cawthorn and Witthuhn, 2008; Kralik et al., 2010; Luo et al., 2010; Nocker et al., 2007a, 2007b; Pan and Breidt, 2007; Rogers et al., 2008; Wahman et al., 2009). Additionally, PMA-qPCR has been successfully applied to the study of viable fungi in air and water (Vesper et al., 2008), *Cryptosporidium parvum* oocysts (Brescia et al., 2009), and recently also to enteric viruses (Parshionikar et al., 2010) and bacteriophage T4 (Fittipaldi et al., 2010).

The aim of this study was to optimize PMA treatment for selective qPCR detection of live *L. pneumophila* cells in regard to PMA concentrations and light exposure times and to apply these parameters to different environmental water samples known for sporadic *Legionella* occurrence. The study furthermore addresses the cytotoxicity of PMA on live cells in comparison with EMA.

2. Materials and methods

2.1. Bacterial strains and cultivation

Legionella pneumophila NCTC 11192 (National Collection of Type Cultures, Colindale, London, UK) was grown on Buffered Charcoal – Yeast Extract (BCYE) containing 0.1% α -ketoglutarate, adjusted to pH 6.9 with KOH, and supplemented (per liter) with 0.4 g L-cysteine and 0.25 g ferric pyrophosphate, 3 g glycine, 1 mg vancomycin, 50,000 IU polymyxin B, and 80 mg cycloheximide (all values per liter). Inoculated plates were incubated for 4-7 days at 37°C in a humidified atmosphere containing 5% carbon dioxide.

2.2. Sample Preparation and Analysis

Legionella cells were typically grown to the mid-exponential growth phase and harvested by centrifugation (10,000 xg for 5 min.). Cell pellets were resuspended in peptone water, followed by serial dilution in peptone water in steps of 10-fold. Samples comprised volumes of 500 μ L. To kill cells, aliquots were exposed to 72°C for 15 min using a standard laboratory heat block (Thermostat plus, Eppendorf) and placed

immediately on ice. The absence of culturable cells was verified by spreading 50 μ L onto BCYE- α plates followed by incubation at 37°C as described previously.

2.2.1. Culture isolation

To determine cell concentrations, one mL of the diluted samples were filtered in triplicate through cellulose membranes (0.45- μ m pore size and 47-mm diameter). Membranes were placed aseptically onto the BCYE- α plate and incubated as previously described. The concentrations of microorganisms in the initial bacterial suspensions were calculated from the plates containing between 10 and 100 colonies, and the weighted averages of log-transformed counts of three replicates (ISO 8199) were expressed in CFU mL⁻¹.

2.2.2. Defined mixtures of live and dead cells

The effectiveness of PMA treatment was further evaluated with mixtures containing different known numbers of live and heat-killed *L. pneumophila* cells. Initial culturable cell numbers were quantified by plate counting. Cell cultures were subjected to serial dilution to obtain suspensions containing 2, 3, 4, and 6 logs of cells. Finally, defined mixtures containing 250 μ L of different concentrations of viable cells and 250 μ L of different concentrations of killed cells were prepared.

2.3. *Natural samples*

To study the effect of different water matrices on the different quantification methods, 125 different cooling tower water samples and 40 ‘clean water’ samples were collected. Sources of ‘clean water’ comprised spas, hotels, hospitals, and tap water, 10 samples were taken from different sites of each source.

Sampling and transport to the laboratory was performed following the ISO 11731 protocol. In the laboratory, 1 L of sample was filtered through 0.4 μm pore-size polycarbonate membranes (Millipore, Molsheim, France). Membranes were subsequently placed in 12 mL of sterile deionized water in a screw-cap tube, and retained cells were released by vortexing for 3 min. Finally, the obtained cellular suspensions were concentrated to approximately 1.5 mL using Amicon Ultra-15 filters (Millipore, Molsheim, France). The resulting volume was divided in three identical fractions, the first fraction was spread onto a BCYE- α agar plate and the second and third fraction were analyzed by qPCR and PMA-qPCR, respectively.

2.4. *PMA treatment*

PMA (Biotium, Hayward, CA) was dissolved in 20% dimethyl sulfoxide (DMSO) to obtain a stock concentration of 20 mM and stored at -20°C in the dark. A total of 1.25 μL of PMA solution was added to 500 μL of sample in a 1.5-mL light-transparent microcentrifuge tube (final PMA concentration of 50 μM). After 5 min incubation in the dark with occasional mixing, samples were exposed to light for 2 or 5 min using a 500-W halogen light source (Fenoplástica, Barcelona, Spain). The sample tubes were subsequently placed horizontally on ice (to avoid excessive heating during light exposure

and to maximize light exposure) in a distance of approximately 20 cm from the light source. Occasional shaking was also performed to guarantee homogeneous light exposure.

2.5. Cytotoxic effect of PMA and EMA

EMA (Molecular probes, Inc. Oregon) was dissolved in 20% dimethyl sulfoxide (DMSO) to obtain a stock concentration of 20 mM and stored at -20°C in the dark. The final concentrations of 100 μ M and 200 μ M were tested with a suspension of 5-log of live *L. pneumophila* NCTC11192. Different aliquots were preconditioned at 4 different temperatures (4, 22, 35, and 44°C) for 2 h and were equilibrated at room temperature before addition of EMA or PMA. Samples were exposed to light as described in section 2.4. Cells were pelleted by centrifugation and resuspended in new medium to get rid of non-incorporated dyes. Non-treated cells served as controls. Finally, cells were serially diluted and 100 μ L of the dilutions were spread onto BCYE- α plates and incubated as previously described.

2.6. DNA isolation and PCR

Cell pellets from 500 μ L of PMA-treated and non-treated samples were resuspended in 50 μ L of 20% Chelex 100 resin (Bio-Rad Laboratories, Richmond, CA.),

followed by three freeze-thaw cycles (-75°C for 10 min and 80°C for 10 min) to lyse cells and to release their genomic DNA. Cellular debris was removed by centrifugation at $10,000 \times g$ for 1 min.

DNA was PCR-amplified in optical microplates using a total volume of 25 μ L. Reaction mixtures contained 1 $\times$ TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA), 300 nM of each *L. pneumophila*-specific primers *dotAF* and *dotAR* (amplifying a 80-bp *dotA* fragment), and 250 nM Taq-Man Minor Groove Binding (MGB) *L. pneumophila*-specific probe labelled with 6-carboxyfluorescein (FAM) (Yáñez et al., 2005). To detect PCR inhibitors, an internal positive control (IPC; described previously by Yáñez et al., 2005) that is amplified simultaneously with the target DNA by the same primer set, was added to each reaction. Amplification was performed using an ABI Prism 7500 sequence detector (Applied Biosystems, Foster City, CA). The thermal profile for both designs was 2 min at 50°C (activation of UNG), 10 min at 95°C (activation of the AmpliTaq Gold DNA polymerase), followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

2.7. Statistical analysis

Error bars in Figure 1 represent standard deviations from three independent replicates. In Figure 3, the results from environmental samples were statistical analyzed using Statgraphics Plus version 5 (Manugistics, Inc.).

3. Results

3.1. Optimization of the PMA protocol on pure *L. pneumophila* cultures

PMA concentrations and light exposure times were optimized to discriminate live from heat-killed *L. pneumophila* cells in pure cultures. A *L. pneumophila* suspension containing 5.3×10^4 CFU mL⁻¹ was divided in two identical aliquots. One of them represented the untreated control, and the other one was exposed to 72°C for 15 min. Heat treatment resulted in a complete loss of culturability as confirmed by plating an aliquot on appropriate culture medium. A cell suspension of 1,000 CFU mL⁻¹ viable *L. pneumophila* served as growth control and resulted in the expected number of colonies. Samples were exposed to different PMA concentrations followed by light-exposure for either 2 or 5 min.

Compared to untreated controls, PMA treatment of live cells exposed to light for 2 min, reduced qPCR signals by 0.3-logs, 0.4-logs, and 0.5-logs for PMA concentrations of 5 µM, 25 µM, and 50 µM, respectively (Figure 1A). Light exposure for 5 min, on the other hand, resulted in qPCR signal reductions of 0.3-logs, 0.5-logs, and 0.6-logs for PMA concentrations of 5 µM, 25 µM, and 50 µM, respectively. In samples containing 200 µM PMA, a signal reduction of 3-logs was obtained for both light exposure times, although this reduction was a consequence of unspecific dye-induced qPCR inhibition (probably due to residual PMA after DNA extraction), since amplification of the IPC of the PCR reaction was completely inhibited.

In case of the heat-killed cells (Figure 1B), a 2 minute light exposure produced a signal reduction of 1.8-logs, 3.0-logs, and 4.0-logs for PMA concentrations of 5, 25, and

50 μ M, respectively. Increasing light exposure time to 5 min resulted in substantially stronger signal reductions with decreases of 2.4-logs, 3.59-logs and 4.35-logs for the three different PMA concentrations. As seen for live cells, a PMA concentration of 200 μ M resulted in unspecific inhibition as treatment did not only completely abolish the amplification of the target gene (for both light exposure times), but also of the IPC. Taken together, treatment with a PMA concentration of 50 μ M followed by 5 min of light exposure was considered optimal to achieve a compromise between minimal impact on intact cells and at the same time maximal signal reduction for compromised cells.

3.2. Effect of PMA treatment on viable *L. pneumophila*

To investigate the effect of the optimized PMA treatment conditions on live *L. pneumophila* cells, we worked with exponential phase cultures where the great majority of cells can be expected to be viable. Cell numbers were determined from the undiluted ‘stock’ culture or serial 10-fold dilutions thereof using culture isolation, qPCR, and PMA-qPCR (Table 1). Values determined by qPCR were on average 1.7 log units higher than those obtained by culture isolation. Differences between culture isolation and PMA-qPCR were substantially smaller with an average difference of 0.39 log units. In case that the extreme outliers of 0.89 and 1.19 log units were not included in the calculation, the average difference between culture and PMA-qPCR decreased to 0.27 log units. Comparing data obtained from qPCR and PMA-qPCR, PMA treatment resulted in an average reduction of 1.3 log units of calculated *Legionella* cells numbers. The PMA-

induced signal reduction suggested the presence of a certain proportion of membrane-compromised cells in these 'live' cell suspensions.

3.3. Effect of PMA treatment on heat-killed *L. pneumophila* cells

To study the effect of the PMA treatment on dead cells, exponential-phase *L. pneumophila* cultures containing 5×10^5 CFU mL⁻¹, were subjected to heat treatment for 15 min at 72°C. As for untreated live cells, cell numbers were determined from undiluted culture or serial dilutions thereof using culture isolation, qPCR, and PMA-qPCR (Table 2). As expected, heat treatment resulted in complete loss of growth on culture plates. Comparing qPCR and PMA-qPCR, PMA treatment reduced the *Legionella* PCR-determined cell numbers by an average of 4.45-logs. Taking into account the qPCR detection limit of 2.82-logs, no amplification was obtained when the concentration of dead cells was below 5.92-logs (corresponding to 831,000 gu L⁻¹).

3.4. Effects of PMA on defined ratios of viable and dead *L. pneumophila* cells

To assess the efficiency of PMA treatment to limit detection to viable intact cells in the presence of a background of dead cells and in samples containing different cell concentrations, and PCR quantification, defined mixtures containing different ratios of viable-culturable and heat-killed cells were subjected to PMA treatment or not, followed by qPCR quantification. Results are shown in Table 3. Without PMA treatment, the concentration of genome copies obtained by qPCR correlated as expected with the total

concentrations of cells, independent of the live/dead ratio. PMA treatment, on the other hand, consistently resulted in lower Ct values in the presence of dead cells with values being closer to the number of living cells than without PMA treatment. However, the correlation with culturable live cell numbers (meaning with the numbers at the top of the columns) depended on the number of dead cells present in the mixtures and the ratio between live and dead cells. The best correlation with the number of living cells was obtained in the first column with 6.7 log units of live cells. For lower numbers of live cells (meaning for the columns further to the right), the correlations tended to be better with lower number of heat-killed cells whereas increasing number of dead cells tended to result in higher deviation from live cell numbers. This tendency was especially obvious in the presence of 4.7 and 6.7 log units of dead cells, where a strong deviation from live cell numbers was obtained. Overall, the data suggest that the presence of high numbers of dead cells exceeded the capacity of PMA to suppress the PCR signal from those cells, probably because the dye did not reach a sufficiently high concentration in the cells to saturate the DNA in the region targeted by the primers. The results from samples containing only membrane-compromised cells (last column in Table 3) suggested that the limit of signal exclusion was somewhere above 4 log units of dead cells as PMA treatment could suppress the signal from 4.7 logs per mL of dead cells, whereas the presence of 6.7 log per mL of dead cells resulted in a relatively strong qPCR signal (equivalent to 3 logs of cells).

3.5. Natural samples

To investigate the usefulness of PMA-qPCR for the detection of viable *L. pneumophila* in environmental samples, a total of 40 water samples from ‘clean’ environments and from 125 different cooling tower samples were tested for the presence of *Legionella* using culture isolation, PMA-qPCR, conventional qPCR (Table 4).

Clean water environments included spas, hotels, hospitals, and tap water (TW) with 10 samples taken for each of these environments from different sites accounting for 40 samples in total. Of these 40 samples, 23 were negative by all three methods, 12 were positive by all three methods, three were positive by qPCR and PMA-qPCR, and two were positive only by qPCR. Results from samples which tested positive for the presence of *Legionella* with at least one of the three methods are shown in Table 4. With the exception of Spa 7, PMA-qPCR resulted in lower numbers of genomic units than qPCR with values being in better agreement with the *Legionella* numbers determined by culture isolation. Nevertheless the differences between PMA-qPCR and culture isolation varied between samples. In Spa 5, Spa 7, Hotel 1, Hotel 3, Hotel 4, Hotel 5, TW 1, TW 4, and TW 5, the PMA-qPCR results were more than 1 log higher than those obtained by culture isolation, whereas in Spa 1, Spa 6, and Hotel 2 the differences in cell concentrations determined by the two methods were less than 1 log unit. Interestingly, two samples (Spa 2 and Spa 3), which tested negative by culture isolation also tested negative by PMA-qPCR (meaning that the numbers of *Legionella* cells was below the limit of detection of these methods, 1.48-log cfu/L and 2.99-log cfu/L, respectively), whereas qPCR provided a positive value. For samples Spa 4, TW 2 and TW 3, both qPCR and PMA-qPCR gave positive results, whereas determination by culture was negative or, more precisely, below the limit of detection. In general, PMA-induced signal reduction in qPCR might indicate

the presence of membrane-compromised cells, whereas the difference between culture isolation and PMA-qPCR might indicate the presence of intact non-culturable cells.

Out of the 125 cooling water tower (CT) samples, a total of 10 samples tested positive by at least two of the methods (Table 4). In samples CT 1, CT 3, CT 6, and CT 8 the concentration of cells obtained by PMA-qPCR was higher than that obtained by culture isolation indicating that some of the cells in the samples might have been intact, but non-culturable. In samples CT 2, CT 4, and CT 9, the *Legionella* concentration obtained by PMA-qPCR was lower than that obtained by culture isolation meaning that PMA-qPCR underestimated the concentration of viable cells in these samples.

3.6. Cytotoxic effects of EMA and PMA on *L. pneumophila*

L. pneumophila cells were exposed to two different concentrations of PMA and EMA (100 μ M and 200 μ M) after preconditioning exponential cultures at four different temperatures (4, 22, 35 and 44°C) for two hours. Comparisons of plate counts obtained after dye exposure in relation to the counts obtained from non-dye exposed controls are shown in Table 5. Exposure to 100 μ M PMA showed only a very modest cytotoxic effect, which was not greatly influenced by temperature. Slightly stronger cytotoxicity was observed when increasing the concentration to 200 μ M, the strongest effect was seen when preconditioning the cells at 44°C. Exposure to identical concentrations of EMA, on the other hand, revealed a substantially stronger cytotoxic effect of this dye at the

concentrations studied. The cytotoxicity gradually increased for both EMA concentrations when preconditioning the cells to higher temperatures.

4. Discussion

Despite its sensitivity and rapidity, the implementation of qPCR for the direct detection and quantification of *L. pneumophila* in environmental samples is greatly hampered by the method's inability to differentiate between live and dead cells. We assessed in this study the use of PMA combined with qPCR as a potential alternative of plate counting to detect and quantify *L. pneumophila* cells in different water samples. In line with previously published protocols for other bacterial species, a first step consisted in testing different PMA concentrations and light exposure times for detecting *L. pneumophila*. Whereas increasing PMA concentrations from 5 to 50 μM did not affect the qPCR signals from live cells, a substantial signal reduction was seen with heat-killed cells. A dye concentration of 200 μM , on the other hand, resulted in complete elimination of the signal from dead cells, but also in a signal reduction with live cells probably due to unspecific inhibition of PCR amplification as indicated by its impact on the internal positive control. Regarding light exposure time, substantially greater signal reduction from dead cells was obtained by exposing samples for 5 min compared with 2 min, whereas the signal from live cells was not affected by the light exposure time. The light exposure time to achieve optimal efficiency of PMA treatment can be assumed to be directly correlated with the intensity of the bulb in the dye's excitation wavelength range, around 464 nm, and to vary between different light sources. In summary, a dye

concentration of 50 μ M and a light exposure time of 5 min were found optimal with the halogen light source used in this study. These parameters when applied to heat-killed cells resulted in a qPCR signal reduction of more than 4-logs. Similar log reductions were reported for a variety of other gram-negative species suggesting that the method shows a uniform performance across bacterial species.

At the same time, our results from different concentrations of heat-killed cells (Table 2) and defined mixtures of live and heat-killed cells (Table 3) showed that in samples containing more than approximately 4.5 logs of membrane-compromised cells, the qPCR signal was not suppressed entirely by PMA and false-positive results were obtained. The presence of such high numbers of membrane-compromised cells probably exceed the dye's capacity as its concentration within the cells is not sufficient to modify all the DNA in the region targeted by the primers. In this respect the comparison with the alternative viability dye EMA is of interest. Chang et al. suggested in a recent study on *L. pneumophila* (Chang et al., 2010) that EMA has a higher capacity to exclude membrane-compromised cells. Comparing the qPCR signal reductions caused by sample treatment with the two dyes, the authors reported that 4-fold higher concentrations of PMA were necessary to obtain a comparable signal reduction of 5 log units as seen with EMA. A more efficient penetration of EMA into membrane-compromised cells was also observed by the authors of the current study in previous projects (unpublished results). Apart from general differences in membrane permeation properties of the two molecules, this effect might primarily be due to the weaker charge of EMA compared with PMA resulting in higher membrane permeation. More efficient penetration, on the other hand, should result in higher intracellular concentrations, increased saturation of DNA, and more efficient

signal reduction. Assuming a final dye concentration of 50 μM , EMA might have a slightly higher signal reduction capacity of up to 5 log units, whereas the limit of PMA is rather around 4 to 4.5 log units. Applying high PMA concentrations to force a 5 log signal reduction of membrane-compromised cells is unlikely to present a solution as suggested by the unspecific inhibition of amplification of the IPC when treating samples with 200 μM PMA as shown in Fig. 1. Considering the potentially different intrinsic signal reduction limits of the dyes, other approaches will be required than increasing dye concentrations. One solution for end point PCR could lie in the increase of amplicon length as suggested for end point PCR (Luo et al. 2010; Nocker et al. 2010), whereas in qPCR the amplicon size limit has to be considered.

Optimization of treatment with a viability dye does, however, not only depend on the efficiency of the exclusion of signals from membrane-compromised cells, but also depends on the efficient exclusion of the dye from live cells. In contrast to the before-mentioned study performed by Chang et al (2010), we found a substantially stronger cytotoxic effect of EMA in comparison with PMA when exposing live cells to the same concentration of dyes (Table 5). The data suggests that the application of EMA to *Legionella* might suffer from the great drawback of potentially producing false-negative results. This finding is in agreement with previous studies with a range of other bacterial species describing EMA's characteristic to penetrate also intact cells (Flekna et al., 2007; Nocker et al., 2006; Kobayashi et al., 2009; Pan and Breidt, 2007; Rueckert et al., 2005). Also in a previous study by Chang et al. (2009), EMA-qPCR suggested for some environmental samples lower *L. pneumophila* numbers than those determined by plating. The cytotoxic effect of EMA in our study was found to increase when preconditioning

Legionella to increasingly higher temperatures before equilibrating the samples to room temperature and adding the dye. This result was in close similarity to the one found for *Listeria monocytogenes* by Pan and Breidt (2007) who reported a cytotoxic effect for EMA (increasing with the preconditioning temperature), but not for PMA. The result varies from the data found for *L. pneumophila* preconditioned to 4, 25, and 37°C by Chang et al. (2010), who did not find a cytotoxic effect for either dye or temperature in final concentrations of 50 µM (EMA) and 200 µM (PMA). The much less pronounced cytotoxic effect of PMA found in this study makes us feel comfortable to largely ignore the probability of PMA to produce pronounced false-negative results although in some environmental samples slightly lower values were obtained by PMA-qPCR than with culture (Table 4). In other words, we consider the risk of underestimating the number of live cells with PMA small and considerably less than in comparison with EMA. For PMA, this view was supported by the good correlation between PMA-qPCR and plate counting when applying PMA treatment on aliquots of live cells (Table 1). The recent studies applying EMA to *Legionella* used low dye concentrations in the range of approx. 6 to 12 µM (Chang et al. 2010; Delgado-Viscogiosi et al. 2010), which might in part overcome this problem. Low dye concentrations can be assumed to minimize detecting the effects caused by EMA's tendency to enter live cells, whereas EMA concentrations in the range of 24 to 48 µM can result in qPCR numbers lower than the ones estimated by plate counting (Chang et al., 2009). Future research to optimize treatment and analysis parameters will show whether EMA's membrane leakiness interferes with such efforts.

In summary, this study demonstrated that PMA reduces the qPCR signal in samples containing dead *L. pneumophila* with resulting cell numbers correlating substantially better with plate count data compared to qPCR without prior treatment and shows less cytotoxicity than EMA. We consider the resulting lesser probability of underestimating pathogen numbers an important factor in DNA-based diagnostics of *L. pneumophila*. In agreement with previous studies, our data, however, suggest that this signal reduction is limited to a maximum concentration of approx. 4-logs of dead cells. Further investigation will be needed to reduce the PMA-qPCR signal of membrane-compromised cells by one or two additional logarithms.

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References

Anonymous, 2005. European Guidelines for control and prevention of Travel Associated Legionnaires' Disease. 2005. EWLINET and EWGLI. Treatment Methods. http://www.ewgli.org/data/european_guidelines/european_guidelines_jan05.pdf.

Anonymous, 1998. International standard ISO 11731. Water quality-detection and enumeration of *Legionella*., Geneva, Switzerland.

Anonymous, 2005. International standard ISO 8199. Water quality -- General guidance on the enumeration of micro-organisms by culture. International Organization for Standardization, Geneva, Switzerland.

Bae, S., Wuertz, S., 2009. Rapid decay of host-specific fecal Bacteroidales cells in seawater as measured by quantitative PCR with propidium monoazide. Water Res. 43, 4850-4859.

Ballard, A. I., Fry, N.K., Chan, L., Surman, S. B., Lee, J.V., Harrison, T. G., Towner, K. J., 2000. Detection of *Legionella pneumophila* using a real-time PCR hybridization assay. J. Clin. Microbiol. 38, 4215–4218.

Brand, B. C., Hacker, J., 1996. The biology of *Legionella* infection. p. 291-312. In S. H. E. Kaufmann (ed.), Host response to intracellular pathogens. R. G. Landes Company, Austin, Tex.

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.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments Clin. Chem. 55, 611-622.

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

Chang, B., Sugiyama, K., Toshitsugu, 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.

Colbourne, J.S., Dennis, P.J., Trew, R.M., Berry, G., Vesey, G., 1988. *Legionella* and public water supplies. Water Sci. Technol. 20, 5-10.

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.

Dreier, J., Störmer, M., Kleesiek, K., 2004. Two novel real-time reverse transcriptase PCR assays for rapid detection of bacterial contamination in platelet concentrates. J. Clin. Microbiol. 42, 4759-4764.

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.

Fittipaldi, M., Codony, F., Adrados, B., Camper, A.K., Mataró, J., 2010. Viable Real-Time PCR in environmental samples: Can all data be interpreted directly? Microb. Ecol. DOI:10.1007/s00248-010-9719-1.

Hayden, R. T., Uhl, J.R., Quian, X., Hopkins, M.K., Aubry, M.C., Limper, A.H., Lloyd, R.V., Cockerill, F. R., 2001. Direct detection of *Legionella* species from bronchoalveolar lavage and open lung biopsy specimens: comparison of LightCycler PCR, in situ hybridization, direct fluorescence antigen detection, and culture. J. Clin. Microbiol. 37, 2618–2626.

Herpers, B.J., Jongh, B.M., Der Zwaluw, K., Hannen, E.J., 2003. Real-Time PCR assay targets the 23S–5S spacer for direct detection and differentiation of *Legionella* spp. and *Legionella pneumophila*. J. Clin. Microbiol. 41, 4815–4816.

Josephson, K.L., Gerba, C.P., Pepper, I.L., 1993. Polymerase chain reaction detection of nonviable bacterial pathogens. Appl. Environ. Microbiol. 59, 3513-3515.

Junge-Mathys, E., Mathys, W., 1994. Die Legionellose—ein Beispiel für umweltbedingte Infektionen. [Legionellosis—an example of environmentally caused infections.]. Intensiv. 2, 29–33.

Kievits, T., Van Gemen, B., Van Strijp, D., Schukink, R., Dircks, M., Adriaanse, H., Malek, L., Sooknanan, R., Lens, P., 1991. NASBA isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection. *J. Viro. Meth.* 35, 273-286.

Kobayashi, H., Oethinger, M., Tuohy, M.J., Hall, G.S., Bauer, T.W., 2009. Improving clinical significance of PCR: use of propidium monoazide to distinguish viable from dead *Staphylococcus aureus* and *Staphylococcus epidermidis*. **J. Orthop. Res.** 27, 1243-1247.

Kralik, P., Nocker, A., Pavlik, I., 2010. *Mycobacterium avium* subsp. *paratuberculosis* viability determination using F57 qPCR in combination with propidium monoazide treatment. *Int. J. Food Microbiol.* 141, S80-S86.

Luo, J.F., Lin, W.T., Guo, Y., 2010. Method to detect only viable cells in microbial ecology. *Appl. Microbiol. Biotechnol.* 86, 377-84.

Miyamoto, H., Yamamoto, H., Arima, K., Fujii, J., Maruta, K., Isu, K., Shiomori, T., Yoshida, S., 1997. Development of a new seminested PCR method for detection of *Legionella* species and its application to surveillance of legionellae in hospital cooling tower water. *Appl. Environ. Microbiol.* 63, 2489-2494.

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

Nocker, A., Sossa, K.E., Camper, A.K., 2007. Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. *J. Microbiol. Methods.* 70, 252-260.

Nocker, A., Sossa-Fernandez, P., Burr, M.D., Camper, A.K., 2007. Use of propidium monoazide for live/dead distinction in microbial ecology. *Appl. Environ. Microbiol.* 73, 5111-5117.

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

Pan, Y., Breidt, F. Jr., 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.

Parshionikar, S., Laseke, I., Fout, G.S., 2010. Use of propidium monoazide in reverse transcriptase PCR to distinguish between infectious and noninfectious enteric viruses in water samples. *Appl. Environ. Microbiol.* 76, 4318-4326.

Pascual, L., Pérez-Luz, S., Amo, A., Moreno, C., Apraiz, D., Catalan, V., 2001. Detection of *Legionella pneumophila* in bioaerosols by polymerase chain reaction. *Can. J. Microbiol.* 47, 341–347.

Rantakokko-Jalava, K., Jalava, J., 2001. Development of conventional and real-time PCR assays for detection of *Legionella* DNA respiratory specimens. *J. Clin. Microbiol.* 39, 2904–2910.

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

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.

Sabria, M., Campins, M., 2003. Legionnaires disease: update on epidemiology and manage options. *Am. J. Respir. Med.* 2, 235–243.

Soejima, T., Iida, K., Qin, T., Taniai, H., Seki, M., Takade, A., Yoshida, S., 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.

Steinert, M., Hentschel, U., Hacker, J., 2002. *Legionella pneumophila*: an aquatic microbe goes astray. *FEMS Microbiol. Rev.* 26, 149-162.

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.

Wahman, D.G., Wulfeck-Kleier, K.A., Pressman, J.G., 2009. Monochloramine disinfection kinetics of *Nitrosomonas europaea* by propidium monoazide quantitative PCR and Live/dead BacLight methods. *Appl. Environ. Microbiol.* 75, 5555-5562.

Walker, G.T., Fraiser, M. S., Schram, J. L., Little, M.C., Nadeau, J.G., Malinowski, D.P., 1992. Strand displacement amplification—an isothermal, *in vitro* DNA amplification technique. *Nucl. Acids Res.* 20, 1691-1696.

Yáñez, M.A., Barberá, V.M., Catalán, V., 2007. Validation of a new seminested PCR-based detection method for *Legionella pneumophila*. J. Microbiol. Methods 70, 214-217.

Yáñez, M.A., Carrasco-Serrano, C., Barberá, V.M., Catalán, V., 2005. Quantitative detection of *Legionella pneumophila* in water samples by immunomagnetic purification and Real-Time PCR Amplification of *dotA* gene. Appl. Environ. Microbiol. 71, 3433-3441.

Yaron, S., Matthews, K.R., 2002. A reverse transcriptase-polymerase chain reaction assay for detection of viable *Escherichia coli* O157:H7: investigation of specific target genes. J. Appl. Microbiol. 92, 633-640.

Table 1. Comparison of results obtained by culture isolation, qPCR (without PMA treatment), and PMA-qPCR (after prior PMA treatment) for enumeration of serially diluted live *L. pneumophila* cells. PMA-induced qPCR signal reduction is indicated on the right. Cell numbers from qPCR and PMA-qPCR were determined using the following standard curve: $Ct = -3.03 \log_{10} (\text{genomic units}) + 39.61$.

Culture isolation Log(CFU mL ⁻¹)	qPCR Log (gu mL ⁻¹)	PMA-qPCR Log (gu mL ⁻¹)	PMA-induced qPCR signal reduction
7.70±0.03	9.65±0.01	7.86±0.02	1.79
7.74±0.02	9.56±0.01	7.94±0.02	1.62
6.70±0.05	7.98±0.01	6.59±0.03	1.39
6.74±0.06	8.67±0.02	6.94±0.03	1.73
5.74±0.07	7.50±0.01	5.86±0.02	1.64
5.70±0.06	7.43±0.02	5.98±0.02	1.45
4.74±0.07	6.06±0.03	4.89±0.05	1.17
4.70±0.05	6.69±0.02	5.06±0.06	1.63
3.74±0.09	5.35±0.04	4.39±0.07	0.96
3.70±0.08	5.44±0.04	4.09±0.08	1.35
2.74±0.1	3.89±0.06	2.90±0.09	0.99
2.70±0.1	4.65±0.04	3.59±0.09	1.06
1.70±0.11	3.86±0.08	2.89±0.12	0.97

Limit of detection for qPCR and PMA qPCR: 2.82-log gu mL⁻¹ and for culture: 1-log CFU mL⁻¹

Table 2. Comparison of results obtained by culture isolation, qPCR (without PMA treatment), and PMA-qPCR (after prior PMA treatment) for enumeration of serially diluted heat-killed *L. pneumophila* cells. PMA-induced qPCR signal reduction is indicated on the right. Cell numbers from qPCR and PMA-qPCR were determined using the following standard: $Ct = -3.03 \log_{10} (\text{genomic units}) + 39.61$

Culture isolation Log (CFU mL ⁻¹)	qPCR Log (gu mL ⁻¹)	PMA/qPCR Log (gu mL ⁻¹)	PMA-induced qPCR signal reduction
<LOD	9.13±0.02	4.80±0.02	4.33
<LOD	8.90±0.02	5.07±0.01	3.83
<LOD	8.18±0.02	3.59±0.03	4.59
<LOD	8.01±0.02	3.56±0.03	4.45
<LOD	7.80±0.03	2.90±0.03	4.90
<LOD	7.63±0.02	3.07±0.03	4.56
<LOD	5.92±0.03	<LOD	
<LOD	5.77±0.03	<LOD	
<LOD	5.01±0.04	<LOD	
<LOD	4.86±0.03	<LOD	
<LOD	3.96±0.03	<LOD	
<LOD	3.32±0.06	<LOD	
<LOD	2.86±0.05	<LOD	

LOD: Limit of detection

Limit of detection for qPCR and PMA qPCR: 2.82-log gu mL⁻¹ and for culture: 1-log CFU mL⁻¹

Table 3. Effect of PMA on different defined ratios of live and dead *L. pneumophila* cells using qPCR with and without (w/o) PMA treatment.

Dead Cells log(CFU/mL)	Living cells log (CFU/mL)									
	6.7		4.7		3.7		2.7		0	
	W/O PMA	PMA	W/O PMA	PMA	W/O PMA	PMA	W/O PMA	PMA	W/O PMA	PMA
6.7	7.27	6.96	7.15	6.63	7.00	5.38	7.03	4.40	7.50	3.30
4.7	7.85	6.91	6.14	5.87	5.16	4.56	5.17	3.83	5.25	<LOD
3.7	7.94	6.18	5.85	4.90	4.46	4.12	4.16	3.49	4.06	<LOD
2.7	7.62	6.78	5.50	5.03	3.85	3.30	3.13	3.15	3.26	<LOD
0	7.93	6.82	5.79	5.56	4.04	4.14	2.90	3.13	<LOD	<LOD

LOD: Limit of detection: $2.82 \cdot \log \text{ gu mL}^{-1}$

Table 4. Comparison of results obtained by culture isolation, qPCR and PMA-qPCR in water samples which tested positive for the presence of *Legionella* with at least one of the applied methods. Cell numbers obtained by PCR were calculated using standard curves. The two columns on the right show differences between culture/PMA-qPCR (C-A) and between qPCR/PMA-qPCR (B-C).

Sample	Culture isolation Log (cfu L ⁻¹)	qPCR Log (gu L ⁻¹) (B)	PMA-qPCR Log (gu L ⁻¹) C	C-A Log (gu L ⁻¹)	B-C Log (gu L ⁻¹)
Clean waters					
Spa 1	3.18	3.88	3.07	-0.11	0.81
Spa 2	<LOD	3.00	<LOD		
Spa 3	<LOD	3.15	<LOD		
Spa 4	<LOD	4.2	3.65		0.55
Spa 5	2.83	4.22	3.88	1.05	0.34
Spa 6	3.78	4.53	4.12	0.34	0.41
Spa 7	3.18	4.19	4.23	1.05	-0.04
Hotel 1	3.1	3.99	3.72	0.62	0.27
Hotel 2	3.7	4.08	3.83	0.13	0.25
Hotel 3	2.36	4.19	3.28	0.92	0.91
Hotel 4	2.7	4.22	3	0.3	1.22
Hotel 5	2.33	3.28	3.01	0.68	0.27
TW 1	2.36	4.36	3.09	0.73	1.27
TW 2	<LOD	3.25	3.06		0.19
TW 3	<LOD	4.32	3.1		1.22
TW 4	2.33	3.42	3.13	0.8	0.29
TW 5	3.2	6.06	4.2	1	1.86
Cooling tower					
CT 1	4.80	5.30	5.92	1.12	-0.62
CT 2	4.39	5.19	3.80	-0.59	1.39
CT 3	4.25	5.05	4.96	0.71	0.09
CT 4	3.93	4.86	3.29	-0.64	1.57
CT 5	3.55	4.68	3.07	-0.48	1.61
CT 6	2.78	3.72	3.40	0.62	0.32
CT 7	2.60	3.29	3.12	0.52	0.17
CT 8	2.38	3.10	3.00	0.62	0.10
CT 9	2.11	3.09	<LOD		
CT 10	2.10	3.08	3.05	0.95	0.03

LOD: limit of detection

Limit of detection for culture isolation: 1.48-log cfu L⁻¹

Limit of detection for qPCR and PMA-qPCR: 2.99-log cfu L⁻¹

TW: Tap water

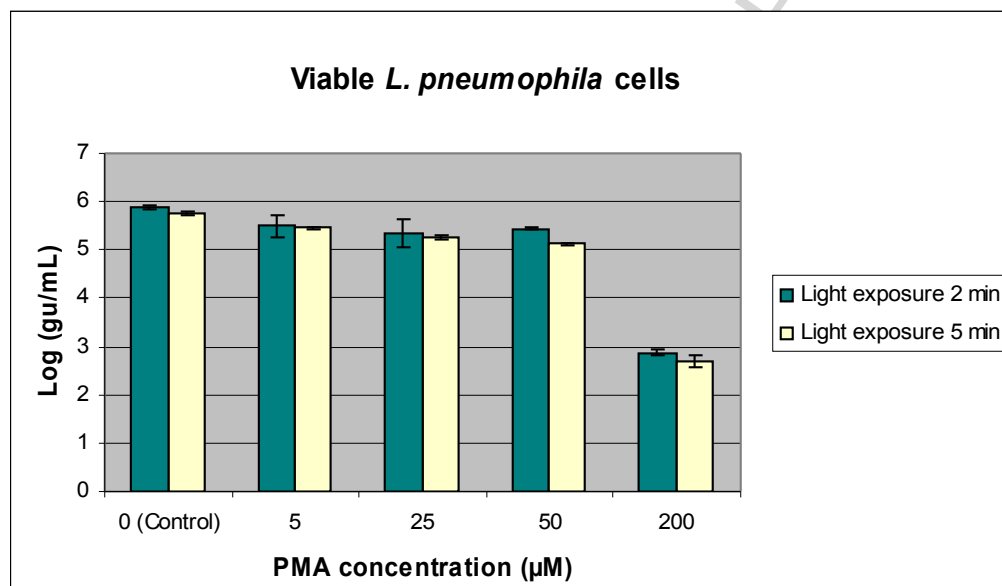
CT: Cooling tower

Table 5. Cytotoxic effect of different concentrations of PMA and EMA on live *L.*

pneumophila cells. Results are expressed as relative difference between log (CFU mL⁻¹) with PMA or EMA with the log(CFU mL⁻¹) of the non-dye exposed controls. Numbers represent technical averages from three different plate count results.

Temperature (°C)	PMA		EMA	
	100µM	200µM	100µM	200µM
4	-0.10	-0.34	-1.40	-1.32
22	-0.16	-0.37	-1.59	-1.60
35	-0.12	-0.30	-2.07	-2.39
44	.005	-0.44	-2.91	-3.63

A)



(B)

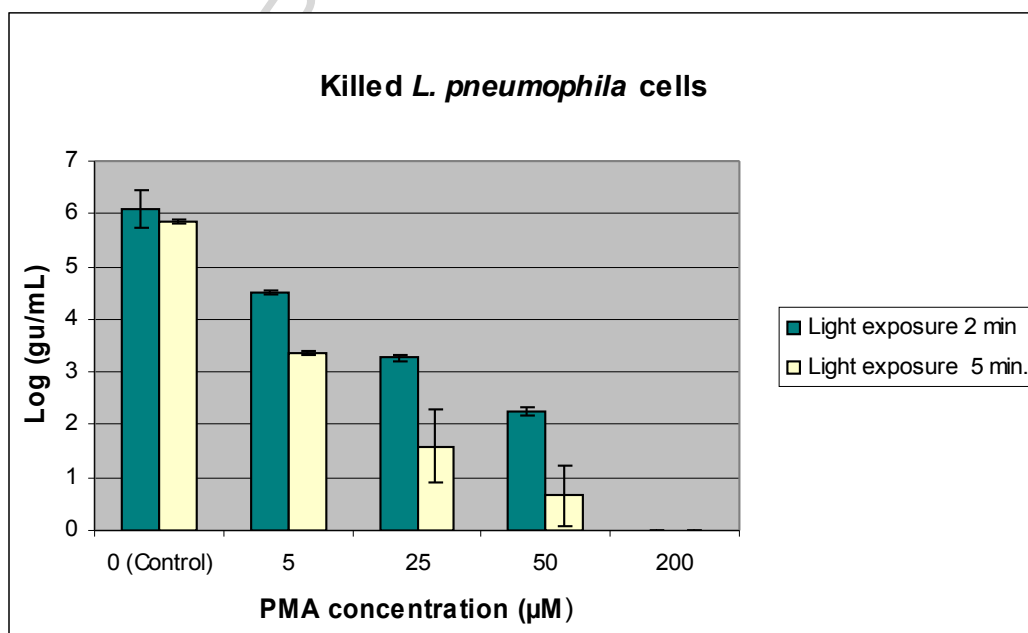


Figure 1. Optimization of PMA concentrations and light exposure times. Cell concentrations obtained by qPCR after exposing live (A) and heat-killed (B) *L.*

pneumophila cells are shown for different PMA concentrations (0, 5, 25, 50 and 200 μM) and different light exposure times (2 and 5 minutes). Error bars in diagrams represent standard deviations from three independent replicates

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