Available online at www.sciencedirect.com
ScienceDirect
Journal of Microbiological Methods 70 (2007) 252–260
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR

Andreas Nocker [a,*], Katherine E. Sossa [b], Anne K. Camper [a,c]

[a] Center for Biofilm Engineering, Montana State University, Bozeman, Montana 59717, United States
[b] Universidad de Concepción, Concepción, Chile
[c] Department of Civil Engineering, Montana State University, Bozeman, Montana 59717, United States

Received 13 March 2007; received in revised form 24 April 2007; accepted 25 April 2007
Available online 1 May 2007

Abstract

One of the major drawbacks of DNA-based microbial diagnostics is its inability to discriminate between live and dead bacteria. Due to the persistence of DNA in the environment after cells have lost their viability, DNA-based assays cannot assess pathogenic risk since signals can originate from both live and dead cells. Presented here is a potential application of the novel chemical propidium monoazide (PMA), which results in the selective suppression of DNA detection from dead cells. PMA can only penetrate dead cells with permeabilized cell membranes. Upon intercalation into the DNA, covalent crosslinkage of PMA to DNA is achieved through light exposure. This modification prevents the DNA from being amplified by PCR. The method, in combination with quantitative PCR as a diagnostic tool, successfully monitored the disinfection efficacy of hypochlorite, benzalkonium and heat on several model pathogens. Threshold cycle numbers increased with increasing disinfection strength after PMA treatment of samples compared to non-PMA treated samples. With some disinfectant-specific differences, monitoring viability loss with membrane integrity as an indicator seemed to be more conservative than monitoring viability loss with plate counts. Loss of viability after short UV-exposure could not be monitored with PMA as UV light affects viability by inducing DNA damage without directly affecting membrane permeability.

Keywords: Propidium monoazide; Live–dead distinction; Disinfection efficacy; Viability; Quantitative PCR

1. Introduction

The reliable detection of viable bacterial pathogens is a great challenge in areas such as water and food safety monitoring, sterility testing of drugs and personal care products, sanitization in the health care setting as well as in the agricultural context. Traditionally, detection is achieved using plate counts or other cultivation-based approaches. As a response to environmental conditions, however, bacteria may not be culturable even though they are still alive, which makes microbial risk evaluation difficult. Moreover, cultivation is time-consuming with positive results often lagging behind the required timeline for preventive measures.

Because of the time required for culturing and the biases that exist in the choice of media and the ability to recover cells in all states of metabolic activity, an increasing number of molecular assays promising fast identification in a real-time manner has been proposed. Apart from other intrinsic biases, one of the major challenges is the differentiation between viable and dead bacteria. Due to the persistence of DNA after cell death (Josephson et al., 1993; Masters et al., 1994), DNA-based quantification can lead to a substantial overestimation of the pathogenic risk or to false-positive results. RNA, on the other hand, degrades rapidly after cells have lost viability (Belasco, 1993), but the same intrinsic instability results in technical problems if used as a molecular target.

Recently, treatment of bacterial samples with propidium monoazide prior to DNA-extraction has been described as a method that allows selective detection of only live cells (Nocker et al., 2006). The principle is based on the integrity of bacterial cell membranes as PMA can only penetrate cells with compromised membranes. Given the lack of more refined viability measurements that can be performed on a routine basis with a larger number of samples, membrane integrity is a well-accepted criterion for distinguishing viable from dead cells (Grégori et al.,

* Corresponding author. Mailing address: Montana State University, Center for Biofilm Engineering, 366 EPS Building, P.O. Box 173980, Bozeman, MT 59717-3980, United States. Tel.: +1 406 994 1849.
E-mail address: anocker@erc.montana.edu (A. Nocker).

0167-7012/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2007.04.014

2001). The exclusion of membrane-impermeant dyes for distinction between viable and dead cells is being used in microscopy (LIVE–DEAD staining kits) and analytical flow cytometry (Barbesti et al., 2000; Grégori et al., 2001). The most commonly used membrane-impermeant DNA-intercalating dye is propidium iodide. In the case of PMA, this fluorescent dye carries an azide group, which allows covalent crosslinkage of the dye to the DNA upon light-exposure. This modification results in signal reduction in quantitative PCR (qPCR) either by the fact that it strongly inhibits PCR amplification or by inducing DNA loss together with cell debris in the subsequent DNA extraction (Nocker and Camper, 2006). The fast and simple treatment of bacterial samples with PMA thus has the potential to limit the analysis to the DNA originating from cells with intact cell membranes.

The aim of this study was to make a first step in evaluating the applicability of the PMA method to monitor killing efficacy using different disinfection methods by comparing the results with plate counting as an alternative viability indicator. Pure cultures of the common human pathogens *Salmonella typhimurium*, *Listeria monocytogenes*, *E. coli* 0157:H7 and *Mycobacterium avium* were used as model organisms. These organisms were paired with disinfection methods that would typically be used for reducing their numbers in specific industrial processes, and included treatment with hypochlorite, benzalkonium chloride (BAC), UV or heat.

Chlorine with its high oxidation capacity is one of the most commonly used antimicrobial disinfection methods in both drinking water and wastewater processing. It is also widely used for surface sanitation in food processing environments (Fukayama et al., 1986) and health care (Rutala and Weber, 1997). Although the mechanism by which chlorine exerts its lethal effect has not been fully elucidated, it is accepted that chlorine exposure of bacterial cells leads to perforation of the cell membrane (Venkobachar et al., 1977).

Benzalkonium chloride (BAC) is a mixture of alkylbenzyl dimethylammonium chlorides of various alkyl chain lengths and is a widely used quaternary ammonium compound (Adair et al., 1969). This synthetic biocide is found in many household cleaners/sanitizers and is used extensively for surface disinfection of the meat and dairy processing environment in the food industry (Krysinski et al., 1992; Merianos, 1991), but also as an antiseptic in medicine (Russell et al., 1986). Quaternary ammonium compounds are known to act on general membrane permeability causing cytolytic leakage at low concentrations and general coagulation in the bacterial cytoplasm at high concentrations (Russell et al., 1999; To et al., 2002).

UV treatment is widely used for drinking water disinfection and is generally considered to exert its lethal action through DNA damage (Sinha and Hader, 2002). Membrane damage might, however, be an indirect consequence of general cell deterioration. Studying the effect of PMA treatment on UV-exposed cells was intended to shed light on the effect of UV on membrane integrity and served as further proof of principle.

Heat disinfection was chosen for its practicality. Killing bacteria or reducing bacterial viability with elevated temperatures might be the oldest and most commonly applied disinfection method. It was shown in a prior study that PMA treatment of bacteria exposed to a lethal dose of heat leads to the selective signal loss of DNA from these cells (Nocker et al., 2006). The heat gradient in the range from sublethal to lethal temperatures studied here was intended to elucidate and refine the sensitivity of the PMA method.

2. Materials and methods

2.1. Hypochlorite disinfection

Salmonella enterica serovar *Typhimurium* (environmental isolate; Department of Microbiology, Montana State University) was grown in Luria Bertani (LB) broth for 12 h at 37 °C in a shaker at 180 rpm. The cell density was adjusted to an OD_{600} of 1 by dilution with LB broth before harvesting cells at 5000 g for 7 min. After removing the medium, the cell pellet was resuspended in an equal volume of phosphate buffered saline (PBS). Increasing volumes of a 1000 ppm sodium hypochlorite stock solution (prepared by diluting 4–6% NaOCl; Fisher Scientific SS290-1) were added to 500 µl resuspended cell aliquots in 1.5 ml microcentrifuge tubes to achieve final concentrations between 5 and 40 ppm. The chlorine stock concentration was measured with a digital chlorine colorimeter kit (DPD method; LaMotte model DC 1100, Chestertown, MD). Identical volumes of a sodium thiosulfate stock solution (1000 ppm) were added after a 15 min incubation (with occasional flipping) resulting in instantaneous dechlorination before harvesting cells (5000 g, 5 min). 40 ppm of thiosulfate was added to a non-chlorine exposed control sample to exclude an effect on viability by this chemical. The supernatant was carefully removed before rinsing the cell pellet with 1 ml of PBS. PBS was carefully removed after another centrifugation step and cells were resuspended in 500 µl PBS before plating serial dilutions on LB agar (overnight incubation at 37 °C) and subjecting sample aliquots to PMA treatment.

2.2. Benzalkonium disinfection

L. monocytogenes (Department of Microbiology, Montana State University) was grown in Brain Heart Infusion (BHI) medium for 12 h (30 °C, 180 rpm) before adjusting the OD_{600} to 1 by dilution with the identical medium. A benzalkonium chloride (Acros Organics, Geel, Belgium) stock solution (5000 ppm) was added to 500 µl culture aliquots to achieve final concentrations in the range between 15 to 60 ppm. Cells were harvested after 30 min incubation by centrifugation (5000 *g*, 5 min). The supernatant was carefully removed and the cell pellets were resuspended in an identical volume of fresh BHI before plating serial dilutions on BHI agar and subjecting sample aliquots to PMA treatment.

2.3. UV disinfection

E. coli 0157:H7 (strain 932; Department of Microbiology, Montana State University) was grown in LB medium for 12 h at 37 °C and standardized to an OD_{600} of 1. 15 ml of cells were harvested by centrifugation (5000 *g*, 5 min) and resuspended in

the identical volume of PBS. The cell suspension was transferred into a 25 mm disposable Petri dish (lid off) and irradiated with short wave UV light (254 nm) using a Spectroline germicidal UV lamp EF 180 (Spectronics Corp., Westbury, NY) placed at 20 cm over the cell suspension. 500 µl aliquots were taken after increasing exposure times and placed on ice until the last sample was taken before plating serial dilutions on LB agar (overnight incubation at 37 °C) and PMA treatment.

To study post-UV membrane integrity under the influence of residual chlorine contained in tap water, *E. coli* 0157:H7 (OD_{600} of 1, prepared as described above) was UV-exposed for 5 min. Two aliquots of 20 ml cell suspension (app. 1.5×10^9 cells/ml) were each filled in a sterile bacterial cage (McFeters and Stuart, 1972) sealed with 0.1 µm Supor®-100 membrane filters (Pall, Ann Arbor, MI, USA) on both sides. Filled cages were submerged in a flow-through reactor with a total volume of 1 l regular tap water and with a water turnover of 1 volume per hour. Residual chlorine concentration was measured as described before. Samples were taken every 24 h for up to 120 h starting at time point zero and either treated with PMA or not, followed by genomic DNA extraction.

To study post-UV membrane integrity under the influence of heat, an *E. coli* 0157:H7 suspension (prepared as before) was UV-treated for 5 min and exposed to heat stress at 58 °C in a regular lab thermo block in 0.5 ml aliquots. Samples were taken every hour from time point zero up to 5 h and were either treated with PMA or not, followed by genomic DNA extraction.

2.4. Heat disinfection

M. avium complex subsp. *avium* (strain W2001; environmental isolate, Boston, Mass., 1998; obtained from Department of Microbiology, Montana State University) was grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) supplemented with 0.2% (w/v) glycerol and 2% Bacto Middlebrook ADC enrichment (Difco) at 37 °C (100 rpm). The OD_{600} was adjusted to 0.3 by dilution with the identical medium. Culture aliquots of 125 µl were transferred into 0.2 ml PCR tubes and exposed to different temperatures for 15 min using a PCR machine (Eppendorf EP gradient). Aliquots were combined to give volumes of 500 µl before PMA treatment and further processing. Serial dilutions of non-PMA treated aliquots were plated on Middlebrook 7H10 agar supplemented with 10% OADC (both from Difco) and 0.5% (w/v) glycerol and grown for app. 10 days at 37 °C.

2.5. PMA cross-linking

PMA (phenanthridium, 3-amino-8-azido-5-[3-(diethylmethylammonio)propyl]-6-phenyl dichloride; Biotium, Inc., Hayward, California) was dissolved in 20% DMSO to create a stock concentration of 20 mM and stored at −20 °C in the dark. 1.25 µl PMA were added to 500 µl culture aliquots to final concentrations of 50 µM in light-transparent 1.5 ml microcentrifuge tubes (Marsh, Rochester, NY, USA; cat. nr. T5015G). Following an incubation period of 5 min in the dark with occasional mixing, samples were light-exposed for 2 min using a 650-W halogen light source (sealed beam lamp, FCW 120V, 3200 K, GE Lighting, General Electric Co., Cleveland, OH, USA). The sample tubes were laid horizontally on ice (to avoid excessive heating) and placed about 20 cm from the light source. After photo-induced crosslinking, cells were pelleted at 5000 g for 5 min prior to DNA isolation.

2.6. DNA isolation and quantification

Genomic DNA was extracted using the Qbiogene soil kit (Qbiogene, Carlsbad, CA, USA). Cell lysis of pure cultures was achieved by bead beating using a FastPrep machine (Qbiogene) for 30 s at a speed setting of 5 m/s. Cell debris was removed by centrifugation at 13,000 *g* for 5 min, both directly after bead beating and after addition of 250 µl of PPS solution (provided in the kit) to the supernatant after bead beating. DNA was eluted with a volume of 120 µl DES buffer (provided by the kit) and visualized on ethidium bromide stained 1% agarose gels. About 10% of the total corresponding eluate volumes were loaded on agarose gels.

2.7. Quantitative PCR

Relative quantitative PCR and data analysis were performed with a SmartCycler II (Cepheid, Sunnyvale, CA). Cycle threshold (C_t) values were automatically calculated by the SmartCycler software using a 30 fluorescence unit threshold. For all experiments, 1 µl of extracted genomic DNA was added to 24 µl of PCR mixture containing Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 10 pmol of each primer. Primers used for quantification are given in Table 1. For amplification of *E. coli* 0157:H7 *stx*1 genes the 25 µl PCR mixture was supplemented with 1.5 µl of 25 mM $MgCl_2$. The cycling parameters for quantification of *S. typhimurium* were: 9 min at

Table 1
Gene targets and primers used for relative quantification of genomic DNA from the bacterial strains

Strain	Gene target	Primer name and sequence (5′–3′)	Product length	Reference
Salmonella sp.	*invA*	invA2-F ATTCTGGTACTAATGGTGATGATC	288 bp	Fey et al. (2004)
	invA	invA2-R GCCAGGCTATCGCCAATAAC		
Listeria sp.	*hly*	lysA-F GGGAAATCTGTCTCAGGTGATGT	106 bp	Guilbaud et al. (2005)
	hly	lysA-R CGATGATTTGAACTTCATCTTTTGC		
E. coli O157:H7	*stx1*	stx1-F ACTGCAAAGACGTATGTAGATTCG	150 bp	Sharma and Dean-Nystrom (2003)
	stx1	stx1-R ATCTATCCCTCTGACATCAACTGC		
Mycobacterium sp.	*treS*	treS-F TACGACACCACCGACCACTA	174 bp	H. Geier (2006, unpublished data)
	treS	treS-R CGTGATCGTCAGAGTCGATG		

95 °C (initial polymerase activation and denaturation) followed by 45 cycles of 20 s at 95 °C, 30 s at 60 °C and 25 s at 72 °C; for *L. monocytogenes*: 9 min at 95 °C followed by 45 cycles of 20 s at 95 °C, 20 s at 55 °C and 15 s at 72 °C; for *E. coli* 0157:H7: 9 min at 95 °C followed by 45 cycles of 20 s at 95 °C, 25 s at 55 °C and 25 s at 72 °C; for *M. avium*: 9 min at 95 °C followed by 45 cycles of 30 s at 95 °C, 20 s at 60 °C and 20 s at 72 °C. For melt curve analysis the temperature was increased in 0.2 °C increments from 60 to 94 °C.

3. Results

3.1. Hypochlorite treatment

Aliquots of a *Salmonella* culture were subjected to increasing concentrations of hypochlorite. Following a 15 min incubation time, the chlorine was inactivated by addition of thiosulfate resulting in instantaneous dechlorination. Plate counts suggested only a minor effect of concentrations of up to 10 ppm (Fig. 1A). Increasing the concentration resulted in a sharp drop in colony counts with concentrations of 25 ppm and higher resulting in complete loss of culturability. A thiosulfate control (with 40 ppm of thiosulfate, but with no hypochlorite added) showed colony counts comparable to the sample with neither chemical added excluding an effect of thiosulfate on culturability. Extraction of genomic DNA from PMA-treated samples showed an increasingly red cell debris pellet for increasing disinfectant concentrations starting at around 25 ppm (Fig. 1B). The most intense red color was visible at a hypochlorite concentration of 40 ppm. The resulting DNA was visualized on an ethidium bromide stained agarose gel: DNA extracted from non-PMA treated cells showed similar yields for all samples whereas PMA treatment resulted in increasingly less visible DNA with increasing concentrations of hypochlorite (Fig. 1C). This tendency was reflected in qPCR detecting differences in invasion A gene (*invA*) copy numbers. Subtracting the C_t values of PMA-treated aliquots from the corresponding non-treated aliquots showed that increasing hypochlorite concentrations resulted in increasingly strong signal reductions (Fig. 1D). Signal reduction started to be significant at a concentration of 20 ppm hypochlorite. The strongest signal reduction of around 14 amplification cycles was observed with 40 ppm hypochlorite treatment.

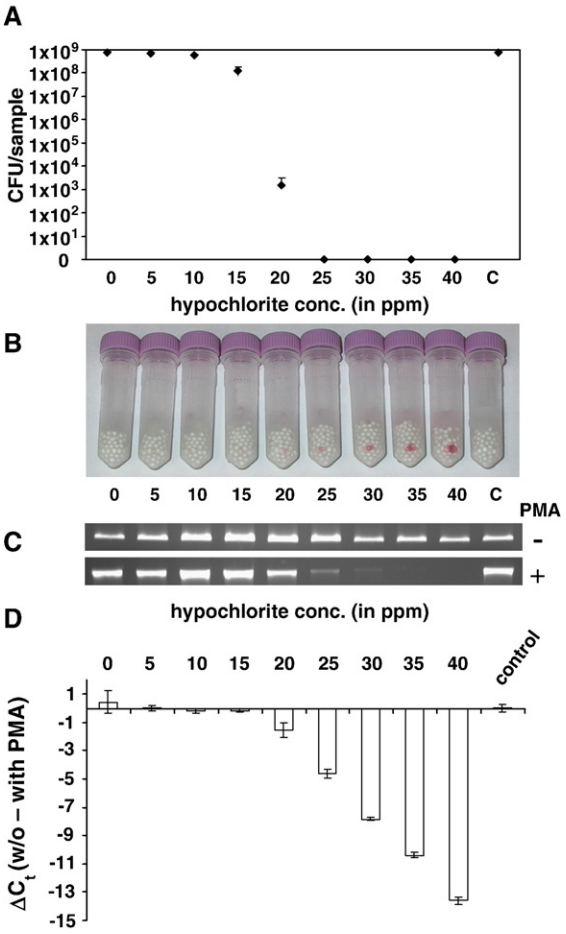

Fig. 1. Monitoring exposure of *Salmonella* to increasing concentrations of hypochlorite (in ppm). Error bars represent standard deviations from three independent replicates. (A) Loss of culturability as determined by plate counts. C stands for the control exposing cells to 40 ppm thiosulfate used for dechlorination of the sample with the highest hypochlorite concentration. (B) Lysis matrix tubes with pelleted cell debris for increasing hypochlorite concentrations after previous PMA treatment. Lysis matrix tubes are shown after centrifugation for 5 min at 14,000 ×*g* with the cell debris facing upward. (C) Genomic DNA from non-PMA treated (top) or PMA treated (bottom) cells as visualized on an agarose gel. (D) Signal reduction as determined by qPCR detecting relative differences in invasin A gene (*invA*) copies. C_t values derived from PMA-treated cultures were subtracted from the corresponding C_t values for untreated cultures.

3.2. Benzalkonium chloride treatment

L. monocytogenes was exposed to increasing concentrations of BAC for 30 min. Concentrations exceeding 30 ppm increasingly reduced culturability (Fig. 2A). No colonies were obtained with concentrations of 55 and 60 ppm benzalkonium. Genomic DNA extraction from PMA treated samples resulted in increasingly red cell debris pellets with increasing disinfectant concentration (Fig. 2B). The loss of culturability was further accompanied by an increased fading of DNA bands on an agarose gel following PMA treatment and DNA extraction whereas DNA band intensities of non-PMA treated cells were not affected by BAC exposure (Fig. 2C). Simultaneously, qPCR detecting differences in listeriolysin O gene (*hly*) copy numbers showed an increasing signal reduction, when comparing C_t values from non-PMA treated and PMA treated aliquots (Fig. 2D). The greatest signal reduction of around 17 PCR cycles was obtained with 60 ppm BAC.

3.3. UV exposure

Exposure of *E. coli* 0157:H7 to UV light, under the conditions chosen, led to a complete loss of culturability within 5 s. This short UV exposure time did not, however, affect PMA uptake by these cells (compared to non-exposed cells) as indicated by the white color of pelleted cells. C_t values after PMA treatment compared with identical but non-UV exposed samples detecting differences in Shiga-like toxin 1 gene (*stx1*) copies (data not shown), were identical. Exposure for longer time periods resulted in an increase in C_t values for both PMA treated and non-PMA

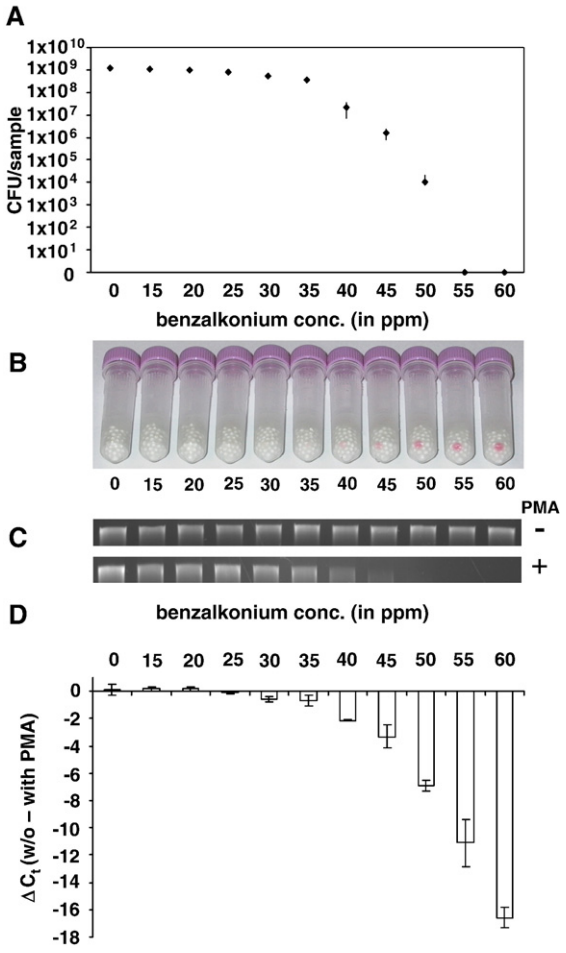

Fig. 2. Monitoring exposure of *Listeria monocytogenes* to increasing concentrations of benzalkonium chloride (in ppm). Error bars represent standard deviations from three independent replicates. (A) Loss of culturability as determined by plate counts. (B) Lysis matrix tubes with pelleted cell debris for increasing benzalkonium concentrations after previous PMA treatment. Lysis matrix tubes are shown after centrifugation for 5 min at 14,000 ×*g* with the cell debris facing upward. (C) Genomic DNA from non-PMA treated (top) or PMA treated (bottom) cells as visualized on an agarose gel. (D) Signal reduction as determined by qPCR detecting relative differences in listeriolysin O gene (*hly*) copies. C_t values derived from PMA-treated culture aliquots were subtracted from the corresponding C_t values of untreated cultures.

treated samples (Fig. 3A). The increasing C_t values correlated with an increased fading of genomic DNA visualized on an agarose gel, again independent of PMA treatment (Fig. 3B). No genomic DNA could be seen on the gel by eye for samples exposed to UV for 30 min and longer.

The facts that PMA treatment of exposed cells was not able to differentiate between culturable and non-culturable cells and that the increase in C_t values with increasing exposure times was independent of PMA treatment, led to the conclusion that cell damage did not result from cell membrane permeability. Only long UV exposures exceeding 45 min resulted in increasingly red staining of PMA-treated cells and presumed membrane damage (data not shown). However, such long exposure times are rarely applied in industry.

To examine whether UV exposure affects membrane integrity post-UV in the presence of other stress factors affecting membranes, untreated (live) and UV-killed *E. coli* 0157:H7 were exposed to tap water containing a residual chlorine concentration of 0.5 ppm. Samples were taken every 24 h for up to 120 h starting at time point zero and were either treated with PMA or not. A linear increase in C_t values over time was observed for both live and UV-killed cells (data not shown). In both cases PMA treatment resulted in increasing C_t value differences over time compared to non-PMA treated samples. In the case of non-UV exposed cells this correlated with a loss of culturability. The fact that increasing PMA uptake (presumably caused by chlorine-induced membrane damage) was independent of UV exposure led to the conclusion that in this experiment the membranes of UV-killed cells did not deteriorate any faster than the ones of non-UV exposed cells. We therefore examined whether subjecting UV-killed cells to heat stress (58 °C) would lead to expedited membrane breakdown compared to non-UV-exposed live cells. Samples were taken every hour starting from time point zero up to 5 h. In this experiment we observed a PMA-induced increase in C_t values for UV-killed cells over time, but not for non-UV exposed cells (data not shown). The difference in C_t values was, however, moderate with an increase of around 2 cycles for the sample heated for 5 h. This finding correlated with the observation that pellets of UV-killed cells were increasingly stained red by PMA whereas pellets of live cells were still white even after 5 h of heat exposure.

3.4. Heat disinfection

Subjecting *M. avium* to increasing heat stress for 15 min resulted in a dramatic loss of culturability around 60 °C (Fig. 4A). No colonies were obtained after exposure to temperatures of 70 °C and higher. This correlated well with a strong increase in qPCR signal reduction detecting differences in trehalose synthase gene (*treS*) copy numbers between 60 °C and 70 °C (Fig. 4B). Temperatures exceeding 70 °C did not lead to any further significant increase of C_t values after PMA treatment. DNA yields obtained from DNA extraction were too low to be visualized on

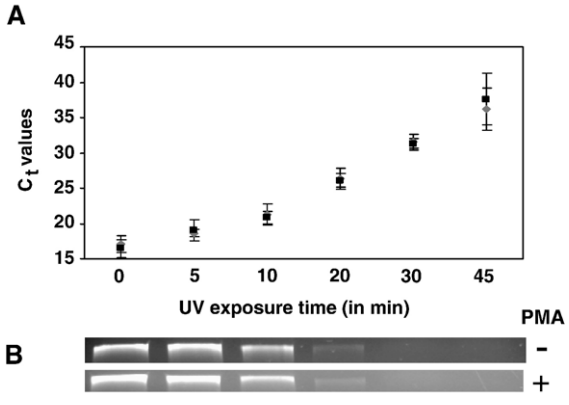

Fig. 3. Monitoring UV-exposure of *E. coli* 0157:H7 for increasing time periods. Error bars represent standard deviations from three independent replicates. (A) Increase of C_t values from PMA and non-PMA treated aliquots determined by qPCR detecting relative differences in Shiga-like toxin 1 gene (*stx1*) copies. (B) Genomic DNA from non-PMA treated (top) and PMA treated (bottom) cells visualized on an agarose gel.

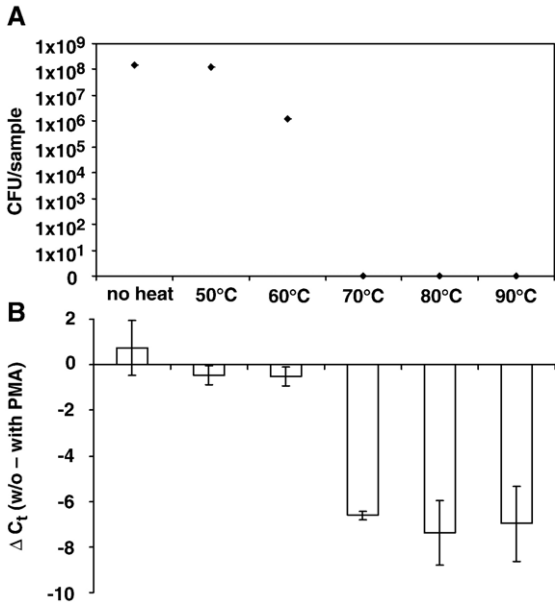

Fig. 4. Monitoring exposure of *Mycobacterium avium* to different temperatures for 15 min. Error bars represent standard deviations from three independent replicates. (A) Loss of culturability as determined by plate counts. (B) Signal reduction as determined by qPCR detecting relative differences in trehalose synthase gene (*treS*) copies. C_t values derived from PMA-treated culture aliquots were subtracted from the corresponding C_t values of untreated cultures.

ethidium bromide stained agarose gels (data not shown). The low DNA concentration might also be the reason why lysis matrix tubes did not show red pellets after centrifugation in the case of PMA-treated heat-killed cells (data not shown).

3.5. Correlation between viability indicators

The plate count data (logarithmic values) were plotted against the qPCR signal reductions up to the disinfectant concentration where for the first time no colonies were counted. The resulting linear correlations showed R^2 values of 0.918 for chlorine treatment, 0.995 for BAC treatment and 0.954 for heat treatment. For increasing chlorine and BAC concentrations the signal reductions increased further, although culturability had already been lost (defined here as non-linear range). The maximal qPCR signal reductions obtained in these experiments were around 13.6 cycles (40 ppm chlorine),16.5 cycles (60 ppm BAC) and 7.0 cycles (90 °C heat).

4. Discussion

The aim of this study was to investigate the applicability of a PMA-based live–dead distinction method to monitor bacterial viability using a selected number of commonly used disinfectants with different killing mechanisms. The effect on culturability was correlated with values obtained from quantitative PCR. Agarose gels with genomic DNA and photographs of lysis matrix tubes serve as supplementary material. For the chlorine and BAC experiments, the decrease of genomic DNA band intensities on agarose gels from PMA treated samples can be explained by the observation that cross-linked dyes like PMA interfere with binding of ethidium bromide to the DNA and therefore interfere with its visualization (Hein et al., 2006). Alternatively, the fading of the bands could result from loss of DNA from killed cells during the extraction procedure together with cell debris. The second hypothesis would gain support if the increasing red color of debris pellets was caused by genomic DNA, but it can't be excluded that the red color is caused by RNA from dead cells. In either case, both the decrease of DNA band intensities on agarose gels and the increasing red color of cell debris can be seen as an indicator for the extent of dye uptake by membrane-compromised cells.

4.1. Chlorine disinfection

As a nonselective oxidant, chlorine can react with a wide variety of cell components. Many different modes of action have been proposed for chlorine's bactericidal effect including the destruction of key metabolic enzymes (Wyss, 1961) and the disruption of protein synthesis (Benarde et al., 1967). Due to its proximity to the environmental stress, the cell envelope also appeared as a plausible site of chemical interaction (Camper and McFeters, 1979). In this study we confirmed that chlorine has a direct impact on cell membrane integrity, therefore allowing the application of PMA to monitor disinfection. However, the concentration of hypochlorite required for maximal qPCR signal reduction exceeded the concentration necessary to reduce cell counts on plates to zero. These observations support a study by Virto et al. (2005) that correlated the effect of chlorine on culturability and membrane permeability. The release of UV-absorbing cytoplasmic substances and the uptake of propidium iodide served as indicators of membrane integrity. Both processes were measurable only after cells had lost their culturability, meaning that there was a discrepancy in chlorine concentrations necessary to inhibit colony formation and to obtain full permeabilization. The described discrepancy was, however, more pronounced than the one seen in this study. A possible explanation could be that propidium iodide was used in a concentration of 2 μg ml^{-1}, whereas in this study the structurally similar PMA was applied in a concentration of app. 25 μg ml^{-1}. Higher dye concentrations might facilitate uptake at a lower degree of membrane damage.

The observation that the hypochlorite concentration required to cause sufficient membrane permeabilization to allow PMA to enter the cells is higher than that required for growth inhibition also correlates with a study looking at the impact of chlorine on various physiological indices in *E. coli* 0157:H7 (Lisle et al., 1999). Using a suite of fluorescent intracellular stains and probes, it was suggested that chlorine affected the indices in the following order: viable plate counts > substrate responsiveness > membrane potential > respiratory activity > membrane integrity. Judging cell 'viability' by membrane integrity as monitored by the PMA assay therefore seems to be at the most conservative end of this scale whereas judging by culturability is at the least conservative end. This observation gains interest as chlorine-caused sublethal injury in coliform microorganisms is suspected to result in underestimation of these indicator organisms in chlorinated water (Barer and Harwood, 1999; Kell et al., 1998; McFeters, 1990). Interestingly, we observed a mixture of normally sized and smaller colonies after

plating cells exposed to 15 and 20 ppm hypochlorite. This colony heterogeneity indicates that injury and recovery might have played a role in the assay. The possibility that some of the non-culturable cells with intact cell membranes might still be viable and might recover under adequate conditions cannot be excluded. Successful resuscitation of permeabilized cells, on the other hand, has never been reported (Nebe-von-Caron et al., 2000).

4.2. Benzalkonium disinfection

Treatment of *L. monocytogenes* with BAC revealed a very good correlation between loss of culturability and loss of membrane integrity as measured by plate counts and signal reduction in qPCR, respectively. In contrast to chlorine, both 'viability' indicators dropped simultaneously with increasing disinfectant concentration. This might be because the mode of action of BAC is primarily based on membrane damage (such as disruption of intermolecular interactions and dissociation of membrane bilayers both compromising permeability control and inducing leakage), whereas in the case of chlorine multiple other, non-membrane related factors might contribute to cell death.

4.3. UV exposure

Whereas the PMA method seems to be able to detect chlorine or BAC-caused membrane damage after disinfection, cell damage caused by UV could not be monitored directly after UV-exposure. This is not surprising considering that the main cellular targets of short wave UV-C light are nucleic acids and not the membrane (Douki and Cadet, 1995; Moan and Peak, 1989). We observed that only long UV exposures exceeding 45 min led to red staining of cells meaning those cells took up PMA more readily than non-UV exposed cells. Such prolonged exposure times obviously led to a breakdown of membrane integrity allowing PMA uptake either as a direct consequence of UV exposure or indirectly through general breakdown of cell integrity. Such long exposure times are, however, unlikely to be relevant in terms of germicidal treatment as cell death can be assumed to occur at much shorter exposure times. In addition, the resulting increased PMA uptake has no or little effect on C_t values in comparison to non-PMA treated samples as the long UV exposure already decreased DNA amplifiability to a minimum.

Interestingly, the increase in C_t values for increasing UV exposure times correlated with increasing loss of fluorescence of the ethidium bromide stained agarose gel. In contrast to the chlorine and BAC experiments, fading band intensity is most probably due to DNA damage including strand breaks and cross-links. Normally the fluorescence of bound ethidium bromide is proportional to the concentration of duplex DNA. It has been reported that ethidium bromide does not bind, however, to pyrimidine dimers (whose formation results in conformational changes), hydrated bases or single-stranded regions caused by UV light (Shaunivan et al., 2001). Therefore the accumulation of DNA damage increasingly prevents intercalation of ethidium bromide into the DNA leading to a loss of fluorescence. This 'ethidium fluorescence assay' has been established for sensitively and conveniently measuring DNA damage caused by radiation (Morgan et al., 1979). The same factors decreasing ethidium fluorescence would also inhibit PCR amplification.

To examine whether UV exposure affects membrane integrity post-UV in the presence of other stress factors affecting membranes, UV-killed *E. coli* 0157:H7 cells were compared to non-UV exposed cells in regard to their resistance to chlorinated tap water and to sublethal temperatures (58 °C). Tap water exposure in a flow-through reactor resulted in comparable cell membrane damage for both UV- and non-UV exposed cells increasing over time. This finding was surprising as faster membrane deterioration for UV-killed cells was expected due to the lack of repair. The heat exposure, on the other hand, resulted in increased PMA uptake over time for the UV-exposed cells compared to non-UV exposed cells. The red staining of the cells was, however, only moderate even after 5 h of heat exposure. This observation was reflected in a moderate qPCR signal reduction of around 2 cycles. Although these results suggested that UV-killed cells are surprisingly resistant to membrane deterioration, this does not exclude the possibility that the simultaneous combination of multiple post-UV stress factors might make them more susceptible to membrane damage due to the lack of repair. Expedited membrane permeabilization would allow the application of the PMA method in post-UV treatment.

4.4. Heat disinfection

Evidence for damage to bacterial membranes by heat has been provided by reports about heat-induced leakage of intracellular substances (Hurst et al., 1974; Russell and Harries, 1967) and loss of lipopolysaccharides (Hitchener and Egan, 1977; Tsuchido et al., 1985). Elevated temperatures have been reported to induce outer membrane blebbing and vesiculation for *Escherichia coli* (Katsui et al., 1982). The heat induced disintegration of membrane structure and permeability barrier function might also be assumed for the inner cell membrane: Heat treatment of *Lactobacillus paracasei* and *Bifidobacterium* sp. in skim milk compromised the cell membrane and allowed the uptake of propidium iodide (Auty et al., 2001). This study confirmed that heat has a direct effect on membrane permeability allowing PMA to enter the cell. A good correlation between loss of culturability and qPCR signal reduction could be observed. The most dramatic change occurred in the temperature range between 60 °C and 70 °C. This is in agreement with a study by Merkal and Crawford (1979) examining heat inactivation of different serotypes of *M. avium* in aqueous suspension. Complete loss of culturability was seen at 70 °C, whereas kill rates were not significant at temperatures of 60 °C or below. In our study elevating the temperature from 60 °C to 70 °C resulted in an increase in qPCR signal reduction of more than 6 cycles whereas the change in signal reduction at temperatures below 60 °C or above 70 °C was only minor.

4.5. Concluding remarks

PMA treatment in combination with qPCR was successful in monitoring the germicidal action of disinfectants directly affecting membrane permeability. This rapid and simple

technology might help to make DNA-based molecular diagnostics more meaningful by primarily detecting cells with intact cell membranes instead of indiscriminately amplifying DNA from all cells including those that are permeabilized. In addition to the disinfection methods described here, PMA uptake and monitoring of killing efficacy by qPCR has also been successfully applied to cells exposed to isopropanol (Nocker et al., 2006). The method is limited, however, to disinfectants causing membrane damage. Although UV-C light does lead to a loss of DNA amplification (probably caused by DNA damage), PMA treatment did not affect C_t values. This does not exclude the possibility that cell death and general loss of cell integrity eventually leads to membrane damage making these cells amenable for PMA uptake.

This study showed that cells exposed to agents that induce membrane damage followed by PMA treatment had reduced qPCR signals. The degree of reduction when compared to plate counts varied between agents. A linear correlation was observed between loss of culturability and qPCR signal reduction for the range in disinfection strengths up to the point where colony counts had dropped to zero. Exceeding this point in disinfection strength led to a further increase in qPCR signal reduction. It remains to be seen which viability indicator (culturability, membrane integrity, or other parameters) correlates best with viability for a certain treatment. In the case of pathogens, the viability indicator which correlates best with infectivity might be most suitable. The potential of PMA for monitoring of other disinfection methods including treatment with hydrogen peroxide, phenol, aldehydes, surfactants, physical pressure or heavy metals like silver and copper and the correlation to traditional cultivation-based screening also remains to be tested. Regardless, PMA treatment shows promise as a rapid, reproducible method for assessing cell integrity after exposure to several disinfectants, and therefore is valuable to those who wish to employ it as an alternative to plate counts.

Acknowledgements

This research has been supported by a grant (DAAD 19-03-1-0198) from the Army Research Office, overseen by Sherry Tove, Chief, Microbiology and Biodegradation, Life Sciences Division. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the Army Research Office. Primers for amplification of the *M. avium treS* gene were kindly provided by Henriette Geier, Microbiology Department, Montana State University.

References

Adair, F.W., Geftic, S.G., Gelzer, J., 1969. Resistance of *Pseudomonas* to quaternary ammonium compounds. I. Growth in benzalkonium chloride solution. Appl. Environ. Microbiol. 18, 299–302.

Auty, M.A.E., Gardiner, G.E., McBrearty, S.J., O'Sullivan, E.O., Mulvihill, D.M., Collins, J.K., Fitzgerald, G.F., Stanton, C., Ross, R.P., 2001. Direct in situ viability assessment of bacteria in probiotic dairy products using viability staining in conjunction with confocal scanning laser microscopy. Appl. Environ. Microbiol. 67, 420–425.

Barbesti, S., Citterio, S., Labra, M., Baroni, M.D., Neri, M.G., Sgorbati, S., 2000. Two and three-color fluorescence flow cytometric analysis of immunoidentified viable bacteria. Cytometry 40, 214–218.

Barer, M.R., Harwood, C.R., 1999. Bacterial viability and culturability. Adv. Microb. Physiol. 41, 93–137.

Belasco, J., 1993. mRNA degradation in prokaryotic cells: an overview. In: Belasco, J., Brawerman, G. (Eds.), Control of Messenger RNA Stability. Academic Press, Inc., San Diego, Calif, pp. 3–12.

Benarde, M.A., Snow, W.B., Olivieri, V.P., Davidson, B., 1967. Kinetics and mechanism of bacterial disinfection by chlorine dioxide. Appl. Microbiol. 15, 257–265.

Camper, A.K, McFeters, G.A., 1979. Chlorine injury and the enumeration of waterborne coliform bacteria. Appl. Environ. Microbiol. 37, 633–641.

Douki, T., Cadet, J., 1995. UV and nucleic acids. In: Jörnvall, H., Jollès, P. (Eds.), Interface Between Chemistry and Biochemistry. Birkhäuser, Basel, pp. 173–197.

Fey, A., Eichler, S., Flavier, S., Christen, R., Höfle, M.G., Guzman, C.A., 2004. Establishment of a real-time PCR-based approach for accurate quantification of bacterial RNA targets in water, using *Salmonella* as a model organism. Appl. Environ. Microbiol. 70, 3618–3623.

Fukayama, M.Y., Tan, H., Wheeler, W.B., Wei, C.I., 1986. Reactions of aqueous chlorine and chlorine dioxide with model food compounds. Environ. Health Perspect. 69, 267–274.

Grégori, G., Citterio, S., Ghiani, A., Labra, M., Sgorbati, S., Brown, S., Denis, M., 2001. Resolution of viable and membrane-compromised bacteria in freshwater and marine waters based on analytical flow cytometry and nucleic acid double staining. Appl. Environ. Microbiol. 67, 4662–4670.

Guilbaud, M., de Coppet, P., Bourion, F., Rachman, C., Prévost, H., Dousset, X., 2005. Quantitative detection of *Listeria monocytogenes* in biofilms by real-time PCR. Appl. Environ. Microbiol. 71, 2190–2194.

Hein, I., Flekna, G., Wagner, M., Nocker, A., Camper, A.K., 2006. Possible errors in the interpretation of ethidium bromide and PicoGreen DNA staining results from ethidium monoazide-treated DNA. Appl. Environ. Microbiol. 72, 6860–6861.

Hitchener, B.J., Egan, A.F., 1977. Outer-membrane damage in sublethally heated *Escherichia coli* K-12. Can. J. Microbiol. 23, 311–318.

Hurst, A., Hughes, A., Collins-Thompson, D.L., Shah, B.G., 1974. Relationship between loss of magnesium and loss of salt tolerance after sublethal heating of *Staphylococcus aureus*. Can. J. Microbiol. 20, 1153–1158.

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

Katsui, N., Tsuchido, T., Hiramatsu, R., Fujikawa, S., Takano, M., Shibasaki, I., 1982. Heat-induced blebbing and vesiculation of the outer membrane of *Escherichia coli*. J. Bacteriol. 151, 1523–1531.

Kell, D.B., Kaprelyants, A.S., Weichart, D.H., Harwood, C.R., Barer, M.R., 1998. Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. Antonie Van Leeuwenhoek 73, 169–187.

Krysinski, E.P., Brown, L.J., Marchisello, T.J., 1992. Effect of cleaners and sanitizers on *Listeria monocytogenes* attached to product contact surfaces. J. Food Prot. 55, 246–251.

Lisle, J.T., Pyle, B.H., McFeters, G.A., 1999. The use of multiple indices of physiological activity to access viability in chlorine disinfected *Escherichia coli* O157:H7. Lett. Appl. Microbiol. 29, 42–47.

Masters, C.I., Shallcross, J.A., Mackey, B.M., 1994. Effect of stress treatments on the detection of *Listeria monocytogenes* and enterotoxigenic *Escherichia coli* by the polymerase chain reaction. J. Appl. Bacteriol. 77, 73–79.

McFeters, G.A., 1990. Enumeration, occurrence, and significance of injured indicator bacteria in drinking water. In: McFeters, G.A. (Ed.), Drinking Water Microbiology. Springer-Verlag, New York, pp. 478–492.

McFeters, G.A., Stuart, D.G., 1972. Survival of coliform bacteria in natural waters: field and laboratory studies with membrane-filter chambers. Appl. Microbiol. 24, 805–811.

Merianos, J.J., 1991. Quaternary ammonium antimicrobial compounds, In: Block, S.S. (Ed.), Disinfection, Sterilization and Preservation, 4th ed. Lea & Febiger, Malvern, Pa, pp. 225–255.

Merkal, R.S., Crawford, J.A., 1979. Heat inactivation of *Mycobacterium avium–Mycobacterium intracellulare* complex organisms in aqueous suspension. Appl. Environ. Microbiol. 38, 827–830.

Moan, J., Peak, M.J., 1989. Effects of UV radiation on cells. J. Photochem. Photobiol., B Biol. 4, 21–34.

Morgan, A.R., Lee, J.S., Pulleyblank, D.E., Murray, N.L., Evans, D.H., 1979. Review: ethidium fluorescence assays. Part 1. Physiochemical studies. Nucleic Acids Res. 7, 547–569.

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.

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.

Russell, A.D., Harries, D., 1967. Some aspects of thermal injury in *Escherichia coli*. Appl. Microbiol. 15, 407–410.

Russell, A.D., Hammond, S.A., Morgan, J.R., 1986. Bacterial resistance to antiseptics and disinfectants. J. Hosp. Infect. 7, 213–225.

Russell, A.D., Hugo, W.B., Ayliffe, G.A.J., 1999. Principles and Practice of Disinfection, Preservation, and Sterilization, 3rd ed. Blackwell Science, University Press, Cambridge, United Kingdom.

Rutala, W.A., Weber, D.J., 1997. Uses of inorganic hypochlorite (bleach) in health-care facilities. Clin. Microbiol. Rev. 10, 597–610.

Sinha, R.P., Hader, D.P., 2002. UV-induced DNA damage and repair: a review. Photochem. Photobiol. Sci. 4, 225–236.

Sharma, V.K., Dean-Nystrom, E.A., 2003. Detection of enterohemorrhagic *Escherichia coli* O157:H7 by using a multiplex real-time PCR assay for genes encoding intimin and Shiga toxins. Vet. Microbiol. 93, 247–260.

Shaunivan, L.L., Delbaere, L.T.J., Lee, J.S., 2001. Gamma and ultraviolet radiation cause DNA crosslinking in the presence of metal ions at high pH. Photochem. Photobiol. 73, 579–584.

To, M.S., Favrin, S., Romanova, N., Griffiths, M.W., 2002. Postadaptational resistance to benzalkonium chloride and subsequent physicochemical modifications of *Listeria monocytogenes*. Appl. Environ. Microbiol. 68, 5258–5264.

Tsuchido, T., Katsui, N., Takeuchi, A., Takano, M., Shibasaki, I., 1985. Destruction of the outer membrane permeability barrier of *Escherichia coli* by heat treatment. Appl. Environ. Microbiol. 50, 298–303.

Venkobachar, C., Iyengar, L., Rao, A.V.S.P., 1977. Mechanism of disinfection: effect of chlorine on cell membrane functions. Water Res. 11, 727–729.

Virto, R., Mañas, P., Álvaraz, I., Condon, S., Raso, J., 2005. Membrane damage and microbial inactivation by chlorine in the absence and presence of a chlorine-demanding substrate. Appl. Environ. Microbiol. 71, 5022–5028.

Wyss, 0., 1961. Theoretical aspects of disinfection by chlorine. Public Health Hazards of Microbial Pollution of Water, Proceedings of Rudolfs Research Conference Rutgers University, New Brunswick, N.J.