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Use of Propidium Monoazide for Live/Dead Distinction in Microbial Ecology▽

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One of the prerequisites of making ecological conclusions derived from genetic fingerprints is that bacterial community profiles reflect the live portion of the sample of interest. Propidium monoazide is a membrane-impermeant dye that selectively penetrates cells with compromised membranes, which can be considered dead. Once inside the cells, PMA intercalates into the DNA and can be covalently cross-linked to it, which strongly inhibits PCR amplification. By using PCR after PMA treatment, the analysis of bacterial communities can theoretically be limited to cells with intact cell membranes. Four experiments were performed to study the usefulness of PMA treatment of mixed bacterial communities comprising both intact and compromised cells in combination with end-point PCR by generating community profiles from the following samples: (i) defined mixtures of live and isopropanol-killed cells from pure cultures of random environmental isolates, (ii) wastewater treatment plant influent spiked with defined ratios of live and dead cells, (iii) selected environmental communities, and (iv) a water sediment sample exposed to increasing heat stress. Regions of 16S rRNA genes were PCR amplified from extracted genomic DNA, and PCR products were analyzed by using denaturing gradient gel electrophoresis (DGGE). Results from the first two experiments show that PMA treatment can be of value with end-point PCR by suppressing amplification of DNA from killed cells. The last two experiments suggest that PMA treatment can affect banding patterns in DGGE community profiles and their intensities, although the intrinsic limitations of end-point PCR have to be taken into consideration.

Genotypic profiling of environmental samples has become a commonly used molecular methodology in microbial ecology. One of the prerequisites to obtain meaningful data, however, is to limit the analysis to the live portion of the microbial communities. The obstacle for DNA-based techniques is that DNA in the environment can be very stable and can persist for extended periods of time (days to weeks) after cell death (10, 13). For example, marine sediment sand or seawater added to chloroform-sterilized radioisotope-labeled sand showed that DNA from killed organisms was degraded only about 60 to 70% after 14 days (16).

It can be assumed that substantial proportions of microbial communities have lost viability (as a percentage of the total community). Luna et al. (12) concluded from a study of different marine sediments that cells with intact membranes accounted for only 26 to 30% of total bacterial counts, while the remaining cells had compromised membranes and thus represented the dominant fraction based on the uptake of propidium iodide (PI). Studying the bacterial population of a eutrophic river in Germany, Freese et al. (8) found that 24% of the total bacterial population (stained with DAPI [4′,6′-diamidino-2-phenylindole]) was intact, also based on the exclusion of PI. Other studies have reported 50 to 70% intact cells in marine and freshwater systems (9, 20).

Apart from producing community fingerprints from complementary 16S rRNA genes obtained from total RNA that had been subjected to reverse transcription-PCR amplification, several innovative approaches have been developed to link community structure with viability. Flow cytometry has been used to sort viable cells (identified by the direct viable count method), substrate-responsive and nonresponsive cells, and actively respiring 5-cyano-2,3-ditolyltetrazolium chloride-reducing cells from an aquatic ecosystem (4). Genetic diversities of these subpopulations were distinct and were different from that of the total microbial community as determined by denaturing gradient gel electrophoresis (DGGE). They were also clearly different compared to the profiles derived from the culturable community fraction (which was <1% of total cell counts).

Ben-Amor et al. (3) described the separation of a fecal microbial community by fluorescence-activated cell sorting (FACS) after staining the sample with the dyes SYTO9 BC and PI. Three fractions were distinguished and defined as viable (SYTO9 BC-stained), injured (SYTO9 BC- and PI-stained), and dead (PI-stained) cells. Based on both scatter parameters and fluorescence signals, the three subpopulations were sorted and then subjected to DGGE characterization of 16S rRNA gene amplicons. Each subpopulation was reported to produce a complex fingerprint that in all cases was less complex than the one of the total fecal community.

Analogous to DNA-intercalating dyes, fluorescent oligonucleotide hybridization probes that bind to RNA targets can be used to label bacterial cells. Amann et al. (1) successfully used specific phylogenetic probe-conferred fluorescence and FACS to resolve individual target and nontarget bacteria. Alternatively, universal fluorescent RNA probes could be used to enrich the metabolically active or the growing fraction of bacterial communities, with the abundance of RNA related to the

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physiological status of cells (5). Cells with fluorescent signals above a certain threshold could be sorted by FACS prior to subjecting this fraction to community analysis.

All these techniques are excellent approaches to restrict microbial communities to the viable or active subpopulations. One of the obstacles of techniques employing flow cytometry, however, is that the sorting depends on the ability to disperse a community into single cells. These individual cells, contained in small droplets, can then be sorted into different pools on the basis of the emitted signal. Bacteria in many environmental samples, however, form aggregates that are difficult to separate due to strong adhesion of cells to each other or to organic or inorganic particles (2, 6).

The goal of this research was to take an alternative approach by assessing the use of propidium monoazide (PMA), in conjunction with end-point PCR and DGGE, for its potential application in microbial ecology. An advantage would be that the sorting problem with FACS would be overcome, since the DNA of entire samples can be subjected to community analysis. PMA has been shown to be useful for live/dead distinction in combination with quantitative PCR (qPCR) (15) and is structurally identical to PI. Both molecules selectively enter cells with compromised membranes. Once inside the cell, they intercalate into the DNA with high affinity and cause red staining. In contrast to PI, PMA has a light-activatable azide group which allows covalent cross-linkage to organic moieties in its proximity upon light exposure. The covalent binding of the dye to DNA results in strong inhibition of PCR amplification. As a consequence, threshold cycle (C_T) values for qPCR are dramatically higher when DNA from PMA-treated dead cells is amplified than when DNA from non-PMA-treated dead cells is amplified (15).

The validation was attempted by subjecting defined live/dead mixtures of a few bacterial environmental isolates to PMA treatment, followed by spiking of an environmental sample with defined live/dead ratios of a model pathogen before examining the impact of PMA on natural environmental samples.

MATERIALS AND METHODS

Defined mixtures of bacterial environmental isolates. Water samples from the Montana State University duck pond were diluted 10-fold with phosphate buffered saline (PBS) and spread on tryptic soy agar plates. Plates were incubated at room temperature for 5 days. Randomly picked colonies were restreaked. Single colonies were grown in tryptic soy broth in a shaker at 180 rpm at room temperature to obtain pure cultures. These were later identified by sequencing of cloned 16S rRNA genes. The optical density at 600 nm was adjusted to 1 by dilution with tryptic soy broth. Cells in 500-μl aliquots were killed by exposure to isopropanol (final concentration, 70%) for 10 min. Isopropanol was removed by harvesting cells using centrifugation at 5,000 × *g* for 5 min and removing the supernatant. Pellets of killed cells were resuspended in 500 μl of PBS. Aliquots of 125 μl of either untreated or isopropanol-exposed cells of the four different species were mixed to give a final volume of 500 μl.

Spiking of a wastewater treatment plant (WWTP) influent sample with defined ratios of live and dead *E. coli* O157:H7. Wastewater influent samples were collected at the WWTP in Bozeman, MT, in 100-ml aliquots. Bacteria were harvested from the wastewater by centrifugation at 5,000 × g for 10 min. The supernatant was removed by decanting and pipetting, leaving the last 7 ml. After resuspension, 434-μl aliquots were distributed into 1.5-ml microcentrifuge tubes for subsequent spiking. *Escherichia coli* O157:H7 (strain 932, a clinical isolate from an outbreak associated with undercooked hamburgers) was grown to log phase in LB medium at 37°C on a shaker at 280 rpm. The culture was diluted to an optical density at 600 nm of 1 by dilution with LB. Cells were harvested by centrifugation and resuspended in PBS. Aliquots (500 μl) in microcentrifuge tubes were killed by exposure to 75°C for 15 min using a standard laboratory heat block. Diluted or undiluted heat-treated 50-μl aliquots did not produce colonies on agar plates. For the spiking experiment, 10^7 total cells (confirmed by plate counts of 10^{-6} diluted aliquots) of different ratios of live and killed cells (in a volume of 66 μl) were added to the 434 μl of concentrated wastewater to give a total volume of 500 μl.

Selected environmental microbial communities. Three microbial communities, municipal wastewater, estuarine benthic mud, and marine sediment, were used. Water samples were collected from an aerated sludge basin at the Bozeman WWTP in 100-ml aliquots. Bacteria were harvested from the wastewater by centrifugation at 5,000 × g for 10 min. The supernatant was removed by decanting and pipetting, except for the last 3 ml. Aliquots of 500 μl were distributed to microcentrifuge tubes.

Estuarine benthic mud and marine sediment together with saltwater (Gulf Breeze, FL) were collected in 50-ml tubes. Samples were mixed by inverting multiple times before allowing mud particles, stones, and sand to settle for 1 min. One milliliter of the turbid supernatant was transferred to clean tubes, and cells were harvested by centrifugation at 5,000 × g for 5 min. Pellets were resuspended in 1 ml PBS and subsequently split in two 500-μl portions for each sample. One aliquot was subjected to treatment with PMA {phenanthridium, 3-amino-8-azido-5-[3-(diethylmethylammonio)propyl]-6-phenyl dichloride; Biotium, Inc., Hayward, CA} (see below), and the other was not.

Exposure of a water reservoir sediment sample to increasing heat stress. Sediment samples were collected from a non-light-exposed water reservoir. Approximately 1 to 2 ml of sample (settled at the bottom of a 15-ml tube) was suspended in the original water in a total volume of 5 ml by pipetting, vortexing (for 10 s), and sonication (for 5 s using the low setting on a T25 S1 machine from Janke & Kunkel GmbH, Staufen, Germany) for resolution of cell clumps. Identical aliquots of 500 μl were distributed in microcentrifuge tubes and exposed to different temperatures in a laboratory heat block for 15 min.

PMA cross-linking. PMA was dissolved in 20% dimethyl sulfoxide to create a stock concentration of 20 mM and stored at −20°C in the dark. Typically, 1.25 μl of PMA was added to 500-μl culture aliquots to final concentrations of 50 μM. Light-transparent 1.5-ml microcentrifuge tubes (T5015G; Marsh, Rochester, NY) were used. 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 120 V, 3,200 K; GE Lighting, General Electric Co., Cleveland, OH). The sample tubes were placed about 20 cm from the light source and were laid horizontally on ice (to avoid excessive heating). Occasional shaking was performed to guarantee homogeneous light exposure. After photo-induced cross-linking, cells were pelleted at 5,000 × g for 5 min prior to DNA isolation.

Genomic DNA extraction. Genomic DNA was extracted by using an Qbiogene soil kit (Qbiogene, Carlsbad, CA). Cell lysis was achieved by bead beating using a FastPrep machine (Qbiogene) for 30 s at a speed setting of 5.5 m/s. Cell debris was removed by centrifugation at 18,000 × g for 5 min, both directly after bead beating and after the addition of 250 μl of protein precipitation solution (provided in the kit) to the supernatant after bead beating. DNA was eluted with a volume of 120 μl DNA elution solution buffer (provided in the kit).

PCR amplification of 16S rRNA genes. Approximately 5 ng of extracted genomic DNA served as a template for amplification of an internal fragment of the 16S rRNA coding gene using 10 pmol (each) of primers 1070F (5′-ATGG CTGTCGTCAGCT-3′; 7) and 1392R (5′-ACGGGCGGTGTGTAC-3′; 7); 1392R had a GC clamp at its 5′ end (5′-CGCCCGCCGCGCCCCGCGCCCG GCCCGCCGCCCCCGCCCC-3′; 7). PCRs were performed in a total volume of 50 μl containing 1× PCR Master Mix (Promega, Madison, WI). Cycling parameters shared by all experiments were the initial denaturation for 2 min at 95°C; set numbers of cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C; and a 5-min final elongation step at 72°C. The number of amplification cycles varied between experiments, as follows: 28 cycles for the amplification of 16S rRNA from defined four-species mixtures, 30 cycles for DNA amplification from spiked and unspiked WWTP influent samples, 25 cycles for DNA amplification from aerated wastewater samples and 30 cycles for the estuarine benthic and marine sediment samples. For amplification of DNA extracted from a water reservoir sediment sample a two-step approach was chosen: 3 μl from a PCR with 10 cycles was used as template for a second PCR with 25 cycles.

16S rRNA genes from duck pond isolates for cloning and sequencing were amplified using primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′; 11), the universal primer 1392R (5′-ACGGGGCGGTGTGTAC-3′; 11), and about 10 ng of the corresponding genomic DNA as template. The PCR mixture was the same as that described above. The cycling parameters were 2 min at 94°C (initial denaturation); 30 cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C; and a 7-min final elongation step at 72°C.

	Closest phylogenetic neighbor	% similarity	Mixtures ○ live ● killed					
			I	II	III	IV	V	VI
A	*Acinetobacter* sp.	100	○	●	●	○	○	○
B	*Aeromonas salmonicida*	100	○	●	○	●	○	○
C	*Aeromonas culicola*	99	○	●	○	○	●	○
D	*Klebsiella* sp.	99	○	●	○	○	○	●

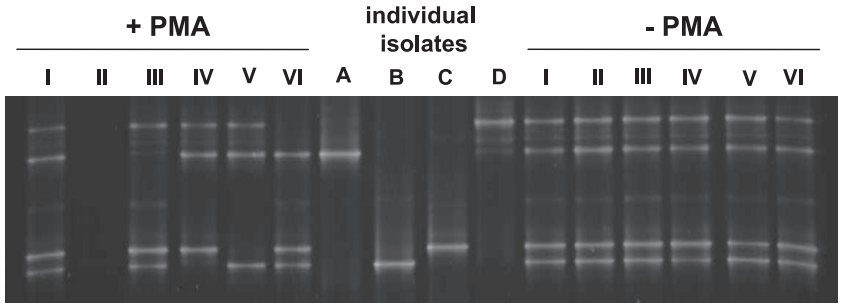

FIG. 1. Effect of PMA on genotypic profiles of defined bacterial mixtures of viable and dead bacterial duck pond isolates. Blast search results of partial 16S rRNA sequences obtained from individual isolates are given together with degrees of similarity to their corresponding nearest phylogenetic neighbor. Identical volumes of untreated or isopropanol-killed pure culture aliquots were mixed according to the scheme given. Mixtures I to VI were either PMA treated (+PMA) or not (−PMA), followed by genomic DNA extraction and amplification of partial 16S rRNA genes. DGGE profiles of PCR products obtained from mixtures are compared to the bands obtained from pure individual isolates.

Cloning and sequencing of 16S rRNA gene PCR products. PCR products obtained from the amplification of individual duck pond isolates were separated on 0.8% agarose gels followed by excision of bands of approximately 1,400 bp in length. The DNA was purified using a QIAquick gel extraction kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. DNA products were cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. Ligation mixtures were used to transform competent *E. coli* TOP10 cells (supplied with the cloning kit). Recombinants were selected by using LB agarose plates containing 50 μg of kanamycin ml^{-1}. *E. coli* transformants carrying plasmids with 16S rRNA gene inserts were grown overnight in LB broth with 50 μg of kanamycin ml^{-1}. Plasmid DNA was isolated using a QIAprep spin miniprep kit (QIAGEN) according to the manufacturer's instructions. Sequencing was carried out at the Genomics Technology Support Facility (Michigan State University, East Lansing, MI) with the M13F-20 primer (5′-GTAAAACGACGGCCAG-3′). Sequences of around 700 bases were entered into the BLAST search program of the National Center for Biotechnology Information for phylogenetic analysis.

DGGE. One hundred- to 150-ng PCR products were analyzed using DGGE. Gels had 8% polyacrylamide and a denaturation gradient of 30% to 70%, where a 100% denaturant is defined as 7 M urea and 40% formamide (all reagents were from Sigma-Aldrich, St. Louis, MO). Electrophoresis was carried out at 60 V for 16 h using a DCode system (Bio-Rad, Hercules, CA). Gels were stained with SYBR Gold (Molecular Probes, Inc., Eugene, OR) and documented using a FluorChem 8800 fluorescence imager (Alpha Innotech, Inc., San Leandro, CA).

qPCR. For relative quantification of *E. coli* O157:H7 used for spiking a wastewater sample, 1 μl of extracted genomic DNA was added to 24 μl of PCR mixture containing 1× Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 10 pmol (each) of primers stx1-forward (5′-GACTGCA AAGACGTATGTAGATTCG-3′; 17) and primer stx1-reverse (5′-ATCTATCC CTCTGACATCAACTGC-3′; 17), and 1.5 mM $MgCl_2$ (in addition to the $MgCl_2$ contained in the Master Mix). qPCR and data analysis were performed with a SmartCycler II (Cepheid, Sunnyvale, CA). C_T values were automatically calculated by the SmartCycler software by using a 30-fluorescence unit threshold. The cycling parameters were 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 melt curve analysis, the temperature was increased in 0.2°C increments from 60 to 95°C.

RESULTS

Profiling of defined mixtures of live and isopropanol-killed environmental isolates. Four bacterial isolates from the duck pond were randomly chosen and cultivated. Partial sequencing of cloned 16S rRNA genes allowed the identification of the closest phylogenetic neighbors (Fig. 1). Identical volumes of either untreated or isopropanol-killed pure cultures were mixed in defined ratios according to the pipetting scheme shown in Fig. 1. Whereas mixture I contained only untreated live cells, mixture II comprised only killed cells. The remaining mixtures comprised three live species and one killed species each. DGGE profiles obtained from partial 16S rRNA gene PCR products for non-PMA-treated mixtures I to VI had four dominant bands reflecting the four isolates. PMA treatment resulted in the suppression of bands of the isopropanol-killed isolates: whereas mixture I (only live cells) still produced four dominant bands, mixture II (only killed cells) did not produce any bands. PMA treatment of mixtures III to VI produced three dominant bands representing the three live species.

Profiling of a WWTP influent sample spiked with defined ratios of live and dead cells. Identical aliquots of a WWTP influent sample were spiked with 10^7 cells of the human pathogen *E. coli* O157:H7 (either untreated or heat killed). Although the total number of *E. coli* O157:H7 cells was constant, the spikes comprised an increasing proportion of live cells, ranging from 0 to 100%. DGGE profiles from amplified 16S rRNA genes are shown in Fig. 2A. The community fingerprint of the WWTP influent sample showed six prominent bands and was not affected by PMA treatment. Each spiked sample without PMA treatment showed an additional band identical in

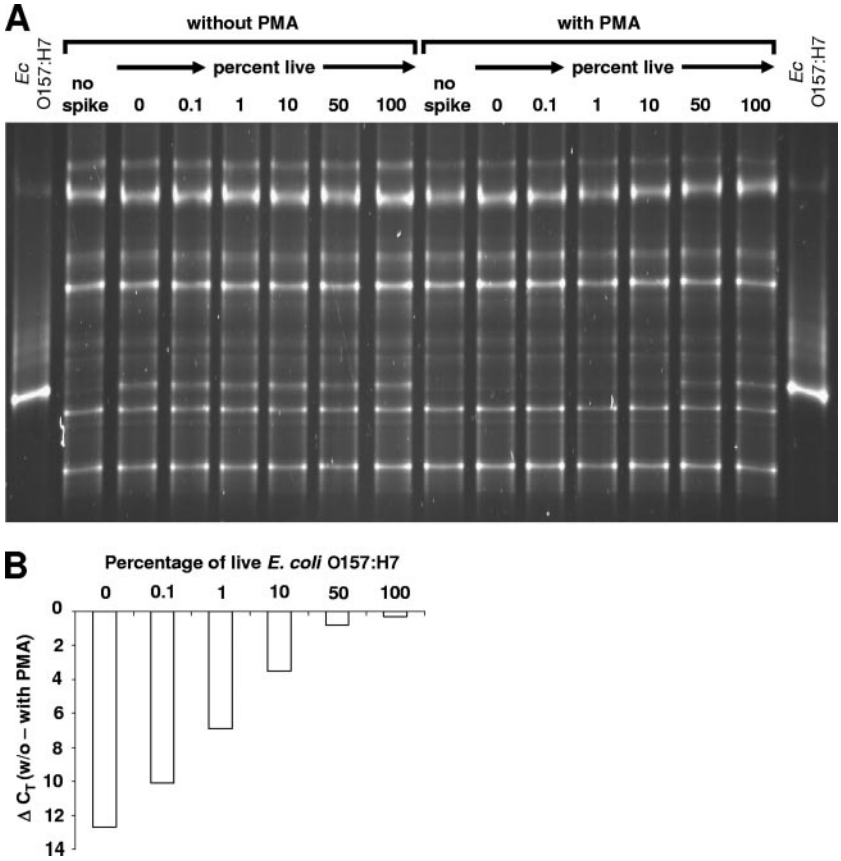

FIG. 2. Analysis of WWTP influent samples spiked with 10^7 *E. coli* O157:H7 cells with increasing proportions of live cells. (A) DGGE profiles of samples that were subjected to PMA treatment or not. 16S rRNA gene profiles from spiked samples are compared to the corresponding profiles from unspiked samples. The band obtained from a pure culture of *E. coli* O157:H7 serves as a reference. (B) Signal reduction as determined by qPCR detecting relative differences in Shiga toxin 1 gene (*stx1*) copies. C_T values derived from PMA-treated spiked samples were subtracted from the corresponding C_T values of non-PMA-treated spiked samples (w/o). The signal reduction shown here is the average for two qPCR runs using the same template.

length to the band derived from a pure *E. coli* O157:H7 culture. Band intensities were comparable independent of the percentage of live cells. PMA treatment led to an elimination of the *E. coli* O157:H7 signal for the samples spiked with 0, 0.1, and 1% live cells. For the PMA-treated sample spiked with 10% live cells, a faint spike band became visible. The band intensity of this pathogen signal became increasingly stronger for samples spiked with 50 and 100% live cells. The intensity of the *E. coli* O157:H7 band of the PMA-treated sample spiked only with live cells was comparable to the intensities of the corresponding bands in the non-PMA-treated samples.

The increasing visibility of the pathogen band with increasing proportions of live cells in DGGE after PMA treatment correlated with the data obtained from qPCR detecting relative differences in Shiga toxin 1 gene (*stx1*) copies (Fig. 2B). C_T values obtained from PMA-treated spiked samples were subtracted from the corresponding C_T values of non-PMA-treated spiked samples. The obtained negative values are referred to as "signal reduction." Signal reduction was strongest with −12.7 cycles for the sample spiked only with dead cells. Increasing proportions of live cells resulted in decreasing qPCR signal reduction. For the sample spiked with only live cells, signal reduction was minimal (−0.3 cycles), meaning PMA treatment had only a minimal effect on the quantification of *stx1* gene copies.

Profiling of selected environmental microbial communities. The results from the previous experiments encouraged us to study the effect of PMA treatment on natural environmental samples. Three diverse communities were chosen: (i) a sample from an aerated sludge basin from a WWTP, (ii) an estuary benthos sample, and (iii) a marine sediment sample. Samples were either PMA treated or not. 16S rRNA gene-based DGGE profiles are shown in Fig. 3. The PMA treatment did not visibly change community profiles of the aerated sludge or the marine sediment sample, but there was one pronounced difference in the estuarine sample, with one prominent band in the non-PMA-treated sample appearing significantly weaker in the PMA-treated sample.

Profiling of a water reservoir sediment sample exposed to increasing stress. A water reservoir sediment sample was chosen to study whether the exposure to heat stress would affect DGGE community profiles. Aliquots of the sample were exposed to 55°C for 15 min and were either PMA treated or not. Resulting DGGE profiles were compared with the ones from the corresponding nonheated samples (Fig. 4). Whereas PMA did not affect the fingerprint of the nonheated sample, it re-

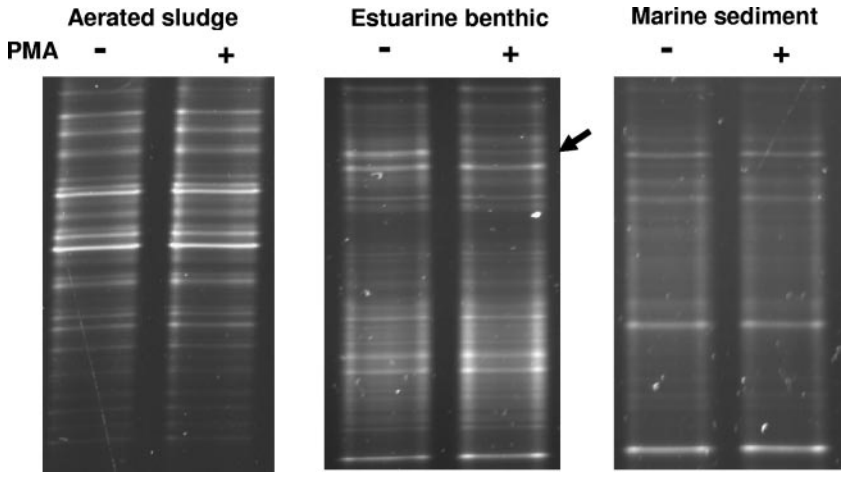

FIG. 3. DGGE profiles of amplified partial 16S rRNA genes of aerated sludge, estuarine benthic, and marine sediment samples without and with prior PMA treatment. The arrow indicates a PMA-caused difference in the estuarine benthic profile.

sulted in loss of some bands in the heat-exposed sample. To confirm this finding, another water sediment sample was exposed to a heat gradient in the range between 45 and 60°C (for 15 min each) followed by PMA treatment. Exposure to 45°C did not affect the DGGE profile compared to either a PMA-treated or non-PMA-treated nonheated sample (Fig. 5). However, increasing temperatures further resulted in increasing loss of DGGE bands.

DISCUSSION

The experiments showed that PMA treatment can efficiently suppress DGGE signals from killed cells in defined mixtures or in an environmental sample spiked with defined mixtures of live and killed cells. PMA also has the potential to significantly alter the assessment of microbial community profiles from samples, which were subjected to heat stress. However, the effect on untreated environmental samples

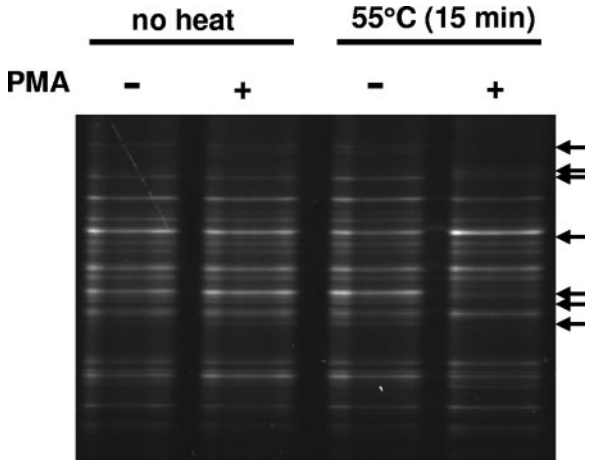

FIG. 4. DGGE profiles of amplified partial 16S rRNA genes of a water sediment sample, which was either not exposed to heat or heated at 55°C for 15 min. Samples were treated with PMA or not prior to DNA extraction and PCR amplification. Arrows indicate the most visible differences in banding patterns.

was either not visible (aerated sludge and marine sediment samples) or it was very small (estuarine benthic sample). There may be several reasons for this observation. It is possible that the method needs modification, potentially by increasing the PMA concentration or the incubation time. However, in the case of the estuarine benthic sample, differences in the DGGE profiles were not more pronounced after the PMA incubation time was increased from 5 min to 15, 30, or 60 min (data not shown). Even a 60-min incubation with a threefold-higher PMA concentration (150 μM) showed no further effect. Another explanation might be the shielding of cells containing PMA from light by particles during the cross-linking procedure. This effect has a low probability, because most solid particles were removed in the sample preparation procedure and samples were slightly shaken to guarantee homogenous light exposure. The most likely explanation lies in the nature of end-point PCR. The amplification of low-abundance templates is increasingly favored with higher cycle numbers. This becomes obvious with qPCR, where the signal intensities of templates with lower initial abundances catch up and finally can reach the same (or even higher) signal levels as templates with higher initial concentrations. One hypothesis to explain this was presented by Suzuki and Giovannoni (19): as the concentrations of product molecules increase, the rate of the bimolecular reaction in which homologous single-strand molecules hybridize with each other will increase as a function of the product concentrations. This reannealing of complementary single strands inhibits the amplification of these templates as single-strand molecules must react with free primer to initiate extension reactions. In reactions with mixed templates, the templates with the higher initial concentrations reach increasingly inhibitory concentrations, while templates with lower initial concentrations continue to undergo efficient amplification. This inherent bias of end-point PCR leads to a mitigation or elimination of differences in gene abundances in the final PCR product pool, which is subjected to community analysis. We speculate that the differences in proportion of live versus dead cells must be very

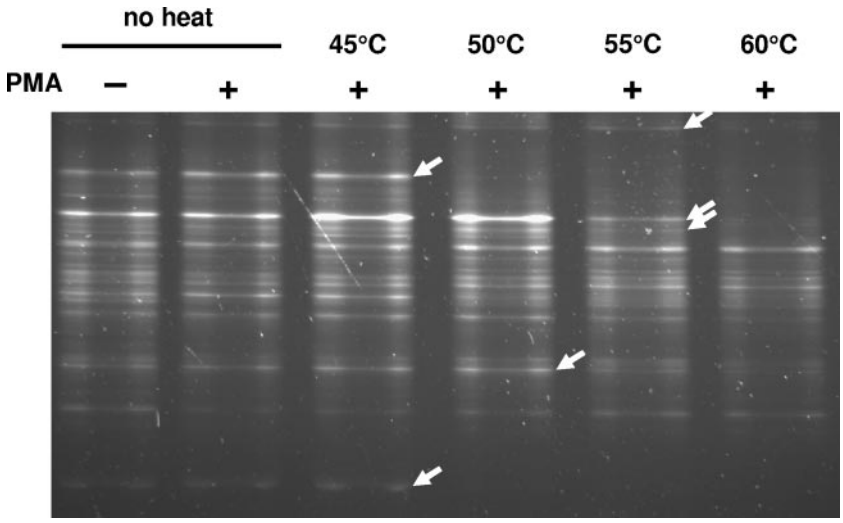

FIG. 5. DGGE profiles of amplified partial 16S rRNA genes of a water sediment sample, which was either not exposed to heat or heated at the indicated temperatures for 15 min. All samples but one were treated with PMA prior to DNA extraction and PCR amplification. Arrows indicate bands which undergo a visible temperature-induced change in intensity in the profiles.

pronounced to be reflected in the final PCR product pool. This might not be the case in many environmental samples. Whether, for example, 30 or 70% of a certain species is membrane compromised is increasingly less likely to be reflected in the PCR products with increasing cycle numbers. Although the spiking experiment suggests that differences can be reflected in DGGE, these differences might have to be great and it might need optimal cycle numbers (or end-points) for visualizing them. It may be that the vast majority of the cells (for example, 99 or 99.9%) of a particular species must be membrane compromised before differences become visible in DGGE patterns.

Community patterns after heat exposure were affected by PMA treatment, possibly because the stress was applied uniformly. Typically, the heat inactivation of bacterial populations is described using first-order kinetics: decimal reduction values (D values) are defined as the time at a given temperature needed to reduce a microbial population by 90%. Although the bacterial population in the sediment sample analyzed in this study and the D values of the individual community members were unknown, we assume that the 15-min heat exposure time had the potential to result in significant viability loss. Existing data support the results by showing that relatively narrow temperature ranges can have a profound effect on bacterial inactivation. A study comparing D values for different waterborne bacteria found in rainwater tanks suggested that the temperature range from 55 to 65°C was critical for effective elimination of enteric/pathogenic bacterial components (18). A substantial temperature effect on D values (determined by enumerating surviving bacteria through plate counting) was seen in this range. At 55°C, a biphasic death curve was observed for *E. coli* with D values of 21 and 4 min; at 60°C and 65°C, D values were 62 s and 3 s, respectively. Results indicated that after 15 min at 60°C, *E. coli* concentrations were reduced by 15 log units. Other waterborne bacteria showed similar or even greater reductions. A rapid heat inactivation in a narrow temperature range was also reported in a study examining heat inactivation of different serotypes of *Mycobacterium avium* in aqueous suspension. Whereas kill rates were not significant at temperatures of 60°C or below, D values at 65°C were 4 min or less (14). Colony formation was no longer observed at 70°C (D value of 1.5 min).

It is critical to note that although PMA treatment of samples shows promise as a method for excluding membrane-compromised cells from analysis, it is no alternative to determining the active fraction of a microbial community. Viable cells may be active or inactive with respect to metabolic activities such as substrate uptake and conversion, respiration, or biogeochemical turnovers. As membrane integrity is no proof of activity, additional techniques are required to further dissect the bacterial fraction with intact membranes. However, as stated earlier, limiting analysis to intact cells would be a step forward in analyzing the more relevant fraction of the total community.

Regardless, PMA treatment of environmental samples may be of benefit in cases where substantial differences in live/dead ratios exist or for monitoring the efficacy of stress or germicidal treatment. Compared to the existing methods using flow cytometry, PMA treatment followed by PCR and DGGE has advantages, because DNA from the entire population can be used and the equipment is more readily available. Additionally, flow cytometry has bias due to cell clumping, and the optimization of the instrument for mixed populations is difficult and tedious. However, due to the limitations of end-point PCR, cycle numbers should be minimized to avoid substantial distortion of template abundances. Currently, there is no method that allows for a precise determination of the overall abundance of community members that produces data comparable to those obtained from qPCR or from fluorescence in situ hybridization for selected targets. The addition of more studies of the PMA effect on environmental samples and comparisons of the corresponding profiles with data from supplementary techniques providing information about viability of community members will help to validate the method further.

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REFERENCES

1. **Amann, R. I., B. J. Binder, R. J. Olson, R. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
2. **Bakker, D. P., B. R. Postmus, H. J. Busscher, and H. C. van der Mei.** 2004. Bacterial strains isolated from different niches can exhibit different patterns of adhesion to substrata. Appl. Environ. Microbiol. **70:**3758–3760.
3. **Ben-Amor, K., H. Heilig, H. Smidt, E. E. Vaughan, T. Abee, and W. M. de Vos.** 2005. Genetic diversity of viable, injured, and dead fecal bacteria assessed by fluorescence-activated cell sorting and 16S rRNA gene analysis. Appl. Environ. Microbiol. **71:**4679–4689.
4. **Bernard, L., C. Courties, C. Duperray, H. Schafer, G. Muyzer, and P. Lebaron.** 2001. A new approach to determine the genetic diversity of viable and active bacteria in aquatic ecosystems. Cytometry **43:**314–321.
5. **DeLong, E. F., G. S. Wickham, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243:**1360–1363.
6. **Dunne, W. M., Jr.** 2002. Bacterial adhesion: seen any good biofilms lately? Clin. Microbiol. Rev. **15:**155–166.
7. **Ferris, M. J., G. Muyzer, and D. M. Ward.** 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl. Environ. Microbiol. **62:**340–346.
8. **Freese, M., U. Karsten, and R. Schumann.** 2006. Bacterial abundance, activity, and viability in the eutrophic river Warnow, northeast Germany. Microb. Ecol. **51:**117–127.
9. **Gasol, J. M., U. L. Zweifel, F. Peters, J. A. Fuhrman, and Å. Hagström.** 1999. Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria. Appl. Environ. Microbiol. **65:**4475–4483.
10. **Josephson, K. L., C. P. Gerba, and I. L. Pepper.** 1993. Polymerase chain reaction detection of nonviable bacterial pathogens. Appl. Environ. Microbiol. **59:**3513–3515.
11. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Chichester, United Kingdom.
12. **Luna, G. M., E. Manini, and R. Danovaro.** 2002. Large fraction of dead and inactive bacteria in coastal marine sediments: comparison of protocols for determination and ecological significance. Appl. Environ. Microbiol. **68:** 3509–3513.
13. **Masters, C. I., J. A. Shallcross, J. A., and B. M. Mackey.** 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.
14. **Merkal, R. S., and J. A. Crawford.** 1979. Heat inactivation of *Mycobacterium avium-Mycobacterium intracellulare* complex organisms in aqueous suspension. Appl. Environ. Microbiol. **38:**827–830.
15. **Nocker, A., C. Y. Cheung, and A. K. Camper.** 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.
16. **Novitsky, J. A.** 1986. Degradation of dead microbial biomass in a marine sediment. Appl. Environ. Microbiol. **52:**504–509.
17. **Sharma, V. K., and E. A. Dean-Nystrom.** 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.
18. **Spinks, A. T., R. H. Dunstan, T. Harrison, P. Coombes, and G. Guczera.** 2006. Thermal inactivation of water-borne pathogenic and indicator bacteria at sub-boiling temperatures. Water Res. **40:**1326–1332.
19. **Suzuki, M. T., and S. J. Giovannoni.** 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. **62:**625–630.
20. **Yokomaku, D., N. Yamaguchi, and M. Nasu.** 2000. Improved direct viable count procedure for quantitative estimation of bacterial viability in freshwater environments. Appl. Environ. Microbiol. **66:**5544–5548.