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Discrimination between live and dead cells in bacterial communities from environmental water samples analyzed by 454 pyrosequencing

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Summary. The preferential detection of cells with intact membranes by sample treatment with propidium monoazide (PMA) in combination with PCR amplification is gaining in popularity. This study evaluates the effect of PMA on 454 pyrosequencing profiles of environmental water samples from a canal in Amsterdam and seawater (with sediment) left untreated or exposed to elevated temperatures (50, 60, or 85°C) for 10 min. Community analysis was based on the extraction of genomic DNA followed by PCR amplification of 16S rRNA genes using universal bacterial primers. Whereas the highest temperature in combination with PMA treatment completely suppressed PCR amplification, PCR products from the other samples were subjected to massively parallel tag sequencing. PMA treatment did not substantially affect the sequence profiles of non-heated samples, but heat exposure resulted in a clear difference in the relative proportions of certain groups. This difference was significantly more pronounced in heated seawater than in heated canal water. The effect of the chosen experimental conditions on the membrane integrity of cells was supported by BacLight LIVE/DEAD staining in combination with flow cytometry, which confirmed an increase in the uptake of propidium iodide in samples exposed to high temperatures. [**Int Microbiol** 2010; 13(2):59-65]

Keywords: live/dead cells distinction · viability · propidium monoazide · 454 pyrosequencing

Introduction

The lack of information on cell viability is a major shortcoming of methods based on DNA analysis. The reason lies in the persistence of DNA for significant time periods after cells have lost their viability, depending on the environmental conditions [7,12]. When a suspension of heat-killed *Salmonella* cells is introduced in a seawater microcosm (initial cell concentration of 10^5–10^6 cells/ml), DNA is detectable for up to 10 days in summer seawater kept at 20°C and up to 55 days in winter seawater kept at 10°C, as measured by PCR [5]. Free DNA spiked into these microcosms persists for a shorter period, but is still detectable for 3–8 days at 10°C and for 2–4 days at 20°C. Similar results were presented by Novitsky [17] regarding the persistence of microbial biomass DNA contained in marine beach sand. Biomass in the sand was labeled with radioisotopes and killed by the addition of chloroform, followed by the addition of untreated sand and/or seawater [17]. The degradation rate of DNA has been estimated at 5–16% per day.

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The percentage of dead cells in environmental water samples can vary greatly. Based on the uptake of membrane-impermeable dyes, Schumann reported that an average of 35–44% cells were membrane-compromised in pelagic water samples from freshwater, estuarine, and coastal stations of the southern Baltic Sea [22]. The number of dead cells can be assumed to vary not only between samples but also between seasons. A higher number of permeabilized cells was found in the above-mentioned study in spring than in winter (35 vs. 44%). In marine surface sediments, approx. 30% of cells stain with ethidium homodimer-2 (EthD-2) [11]. Luna et al. reported an even higher percentage of membrane-damaged cells in a study of propidium iodide (PI) uptake in different marine sediments [10].

To preferentially detect DNA from live cells, treatment of microbial samples with propidium monoazide (PMA) has become an increasingly applied method. The principle is based on membrane integrity as a common sign of viability: PMA does not enter cells with intact membranes, whereas it readily does so in cells with compromised membranes [16]. Once inside dead cells, PMA intercalates with DNA, which is irreversibly modified when the complex is light-activated. Although the exact mechanism is not yet known, DNA modification strongly interferes with PCR amplification of DNA extracted from dead cells treated in this way. At the same time, light exposure inactivates any excess PMA that has not entered cells. Thus, it cannot modify DNA from live cells with intact cell membranes after their DNA is exposed during the extraction procedure. Apart from bacterial applications, the method has also been successful for fungi [26] and the protist *Cryptosporidium* [2].

The treatment of samples with PMA for preferential detection of live cells with intact cell membranes has been used in combination with a number of different downstream analysis tools employing DNA amplification. These include quantitative PCR (PMA-qPCR) [1,14,19], denaturing gradient gel electrophoresis (PMA-DGGE) [15,20], terminal restriction fragment length polymorphism (PMA-TRFLP) [21], and microarray analysis (PMA-microarray) [13]. The present study addressed whether the application of PMA to water samples (left untreated or exposed to elevated temperatures) would result in changes in 454 pyrosequencing profiles. Heat served as an example of stress that causes membrane damage. For this purpose, water samples were collected from a canal in the city of Amsterdam and from a shallow beach at Bloemendaal aan Zee at the North Sea. The marine water samples contained sediment. The observed effects on the 454 sequencing profiles were compared with flow cytometric profiles after the samples had been stained using the LIVE/DEAD BacLight kit.

Materials and methods

Sample collection and processing. Samples were collected from seawater and canal water in the Netherlands on June 15, 2009. Seawater samples were collected at a depth of 0–0.5 m, within 10 m from the coast at Bloemendaal aan Zee, a seaside resort and neighborhood in the municipality of Bloemendaal, in North Holland province (52°23′42″ N, 4°32′16″ E). Canal water samples were collected at a depth of 0–0.3 m at the Rokin, close to Spui square, in the city center of Amsterdam (52°22′8″ N, 4°53′32″ E).

Biological material from 1-liter samples was collected by centrifugation in steps of 250 ml (4000 ×*g* for 15 min), resuspended in approx. 5 ml of remaining water, and split into identical 500-µl aliquots in 1.5-ml microcentrifuge tubes. Two aliquots of each sample were left untreated (kept at room temperature), while the others were exposed to 50, 60, or 85°C for 10 min. All aliquots were treated with PMA (final concentration 50 µM), except one non-heat exposed aliquot of each sample that served as control. PMA treatment consisted of the addition of 12.5 µl of PMA (2 mM) to a final concentration of 50µM, followed by thorough mixing and incubation in the dark for 5 min. The tubes were occasionally inverted to guarantee homogeneous PMA exposure. Samples were placed horizontally on ice and then light-exposed (with occasional tilting) for 3 min using a 650-W halogen lamp (Osram 64553 C318) positioned at a distance of approx. 20 cm from the samples. Biomass was subsequently harvested by centrifugation (5000× *g* for 5 min) followed by extraction of total DNA using the AGOWA mag MINI DNA isolation kit (cat. no. 40401; AGOWA GmbH, Berlin, Germany) according to the manufacturer's instructions.

454 sequencing and data analysis. Extracted genomic DNA was 10-fold diluted to overcome the effect of PCR inhibition. One-µl aliquots of these dilutions were used as template in the amplification of 16S rRNA genes using forward primer 5′-gcctccctcgcgccatcag-GGATTAGATACCCBRGTAGTC-3′ and reverse primer 5′-gccttgccagcccgctcag-tag-TCACGRCACGAGCTGACGAC-3′. Lower-case letters designate the 454-sequencing adaptors attached to the 5′-ends of the primers. The universal primer pair amplifies *E. coli* positions 785–1061 of the 16S rRNA gene, including the highly variable regions V5 and V6. The reverse primer carried a 3-base identification tag. Each 25-µl PCR mixture contained 2.5 µl reaction buffer (10×), 12.5 pmol of each primer, 3 µl dNTPs (2 mM), and 0.5 µl of Pfu Ultra II polymerase (prod. no. 600672, Stratagene). PCR conditions were as follows: initial denaturation of 120 s at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 50°C, and 30 s at 72°C. Identical amounts (100 ng) of PCR products were pooled and sent out for 454 sequencing using GS FLX standard chemistry (Keygene NV, Wageningen, Netherlands). To exclude potential artifacts, only sequences with lengths between 210 and 270 bases (the typical sequence length range for the 454 FLX chemistry used in this study) and with less than two undefined nucleotides were selected for further analysis. Accordingly, between 1566 and 2294 sequences were obtained per sample. Phylogenetic relationships were determined by a BLAST search within the Ribosomal Database Project (RDP) database (release version 10) [3,4]. Sequences were grouped into phylogenetic families and the number of sequences assigned to each phylogenetic family was listed for each sample. The resulting abundance matrix served as the basis for calculating linear correlation coefficients using the PAST software [6]. Abundances of bacterial families of each PMA-treated sample (variable 1) were compared with those in the non-heated and non-PMA treated control sample (variable 2) to calculate *r* values.

BacLight staining. The fluorescent dyes SYTO9 and PI from the LIVE/DEAD BacLight kit (Invitrogen Europe BV, Leiden, Netherlands) were used in combination by mixing identical volumes of 3 µl of each dye and adding water to a final volume of 1 ml. The resulting 2× staining solution was combined with an equal volume of sample. In addition, the two dyes were used alone in identical concentration as in the two-dye mixture.

After dye addition, samples were incubated in the dark for approx. 20 min prior to analysis. Unstained samples served as controls.

Flow cytometry. Flow cytometry measurements were done on an Accuri C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA), with 488-nm excitation from a blue solid-state laser. Green fluorescence was collected in the FL1 channel (530 nm ± 30 nm) and red fluorescence in the FL3 channel (> 670 nm). All values were collected as logarithmic signals. Data collection proceeded until 45,000 counts were obtained for every sample. The minimal values on the FL1 channel were set to 2000 (canal water) and 5000 (seawater), and on the FL3 channel to 1500 for both sample types. Maximal values for the FL1 channel were 5×10^6 (canal water) and 1×10^7 (seawater), and for the FL3 channel 1×10^7 each. Data were analyzed on the CFlow Software provided with the machine. Experiments were performed at least in duplicate and the patterns were reproducible (data not shown).

Results

Genomic DNAs isolated from canal and seawater samples served as templates for PCR amplification of 16S rRNA genes. PCR products were obtained from both non-heated samples and samples exposed to 50 and 60°C. No PCR products were obtained from the samples exposed to 85°C, suggesting that this temperature damaged the membranes of most or all cells contained in the sample and that PMA treatment efficiently inhibited PCR amplification of their DNA within 30 cycles. PCR products from the other samples were sequenced using 454 FLX standard chemistry. The sequences obtained were compared in a BLAST homology search with those deposited in the RDP database to identify their nearest phylogenetic neighbors. Resulting community profiles are schematically represented in Fig. 1, which shows the relative proportions of distinct groups for the different samples.

Whereas no striking PMA effect was seen in samples that were not exposed to heat (compared to the non-PMA treated and non-heated controls), sequence profiles differed in heat-exposed samples. The effect also became obvious following a linear regression analysis, with the differences in sequence patterns reflected by the linear correlation coefficients shown in Table 1. The PMA effect was significantly stronger for seawater samples than for canal water samples.

In seawater, the largest and most obvious effect involved sequences showing close homology to cyanobacteria or chloroplasts of diatoms and large algae. These groups are abundant in marine water and sediments, especially in summer, when this sample was taken, and their ribosomal genes are readily amplified by universal bacterial primers such as the ones used in this study, due to the high sequence similarity of these genes in bacteria and chloroplasts. Another group whose abundance decreased with temperature showed closest homology to Cryomorphaceae. Members of this family belong to the phylum of Bacteroidetes, which are major decomposers of organic matter in seawater [9]. As suggested by the name Cryomorphaceae, these psychrotolerant bacteria would not be expected to withstand high temperatures. By contrast, the relative percentages of Actinomycetales and Caldilineae (phylum Chloroflexi) increased in heat-exposed samples. Actinomycetales are a very heterogeneous group and contain thermophilic members [8]. The same holds true for the bacterial phylum Chloroflexi, as the optimal growth temperatures of some of its members are reportedly in the range of 50–60°C [18,28].

Temperature effects were less pronounced in the canal water sample. As in the seawater sample, the greatest decrease in relative abundance was found in chloroplast sequences, followed by sequences assigned to the group Comamonadaceae, which comprises phenotypically diverse members of the Betaproteobacteria that are common water and soil inhabitants [27]. This reduction in abundance was compensated by an increase in relative abundance of some other groups, including Caldilineae (as in the seawater samples), which appears to consist of heat-resistant members.

The effect of high temperatures and the chosen experimental conditions on membrane integrity was assessed by flow cytometric analysis of samples stained with the LIVE/DEAD BacLight kit (Fig. 2). Whereas no temperature effect was observed in unstained samples or samples stained only with SYTO9 (data not shown), a clear temperature-dependent shift occurred in samples stained with either PI alone or the two dyes in combination. The gates at which temperature-dependent changes were noted for the two water types are highlighted in Fig. 2A and B. The effect increased with increasing temperatures, suggesting that the accumulation of membrane-damaged cells leads to more cells permeable to PI.

Discussion

The results obtained suggest that PMA treatment can change microbial profiles in water samples exposed to high temperatures. Heat was chosen as an example of a stress-causing factor that confers membrane damage. Sequencing results were corroborated by flow cytometry, which showed an increase in the uptake of PI with increasing temperature, suggesting that the experimental conditions affect cell membrane integrity. PMA and PI have probably very similar membrane transfer characteristics due to their structural identity, with the only difference being that, in PMA, an amino group is replaced by an azide group. In accordance with the 454 sequencing data, the effect of high temperatures was stronger in the seawater sample than in the canal water sam-

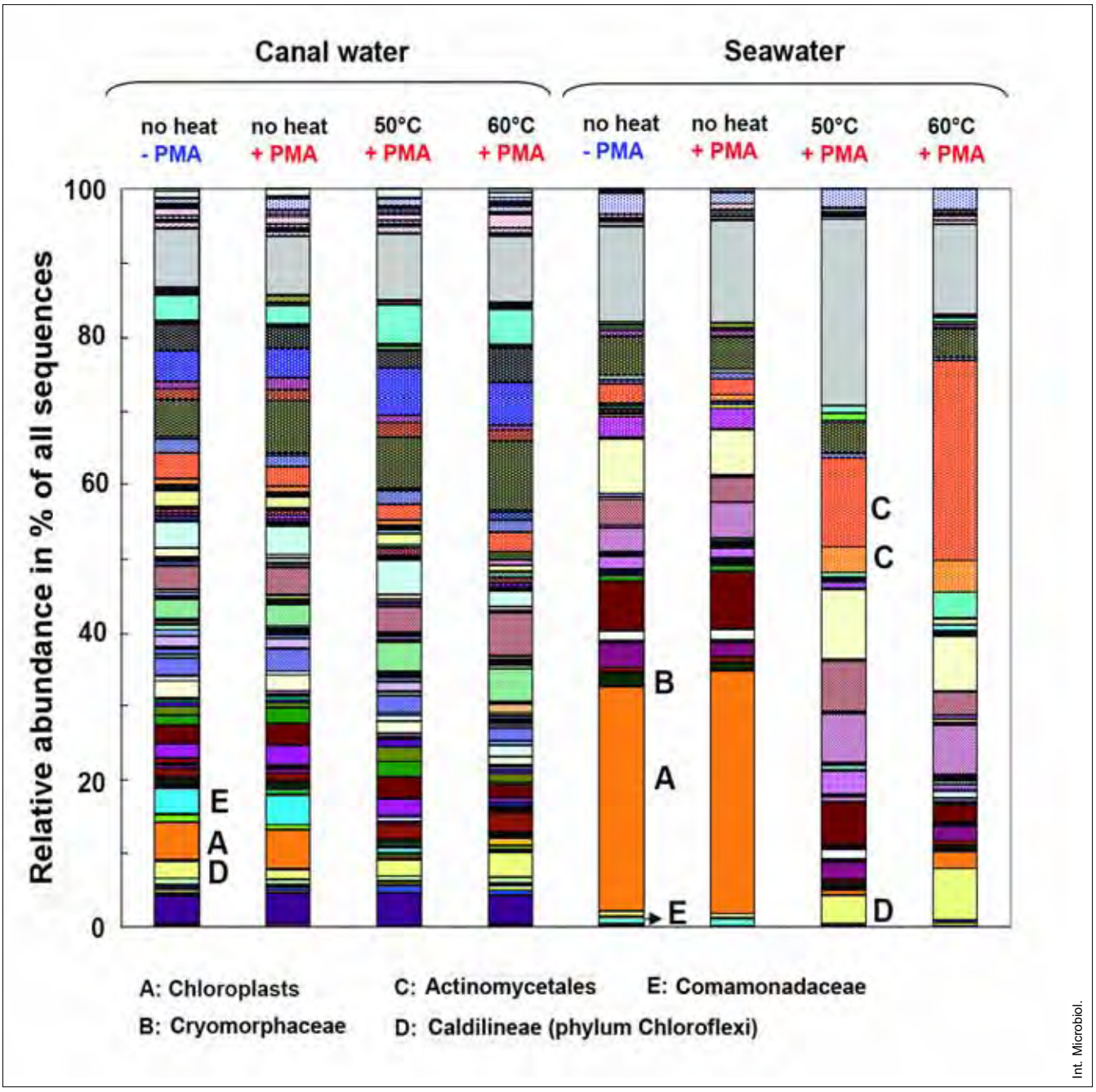

Fig. 1. Non-heated and heat-exposed canal and seawater samples subjected to 454 sequence abundance profiling. All samples were treated with PMA except the non-heated controls. Distinct groups are represented in different colors. Closest phylogenetic neighbors (based on a RDP-BLAST homology search) are indicated for groups undergoing the most visible changes. Both groups designated C show closest homology to Actinomycetales.

ple, as more signals disappeared from the region highlighted in red in Fig. 2.

Although controls for the effect of heat (without PMA treatment) on sequence profiles could not be included in this study due to limitations in sequencing capacity, we believe that the observed effect on 454 profiles can be attributed to PMA treatment rather than to heat treatment. This would agree with previous studies, which have not reported any effect of temperature on the amplification characteristics of DNA from heat-exposed samples. No temperature effect has been observed in heat-exposed *Mycobacterium avium* at threshold cycle values, as measured by qPCR [14]. In another study, in which the suitability of PMA in microbial ecology was assessed, the DGGE profile from a water sediment sam-

Table 1. Linear correlation coefficients calculated from sequence abundance data using PAST software [6] to quantitatively express similarities of sequence profiles. The values refer to the corresponding non-heated samples without prior PMA treatment

	Not-heated + PMA	50°C + PMA	60°C + PMA
Canal water	0.968	0.893	0.840
Seawater	0.995	0.478	0.349

ple exposed to 55°C for 15 min was identical with that from a non-heat-exposed sample [15]. PMA treatment, on the other hand, resulted in an altered DGGE banding pattern in the heat-exposed sample, as was the case in this study for the 454 sequencing patterns.

Despite the effect of PMA on sequencing profiles of samples exposed to high temperatures, the sequencing profiles of non-heat-exposed samples were not changed significantly. This finding is again consistent with data from the above-mentioned previous study employing PMA-DGGE, which reported that PMA treatment of environmental samples such as aerated sludge and marine sediment does not visibly affect microbial profiles [15]. In that study, only in the case of an estuarine benthic sample was a band with a lower intensity upon PMA treatment detected. The reasons for the lack of a significant effect mainly involve the nature of end-point PCR, which tends to minimize differences in initial template abundances with increasing cycle number [25]. It seems improbable that moderate differences in live-dead ratios are still reflected in amplicon ratios after 30 amplification cycles. In the case of the PMA-DGGE study, the authors hypothesized that differences in live-dead ratios have to be substantial to be visible in the final PCR amplicon pools [15]. Pronounced relative differences between intact and mem-

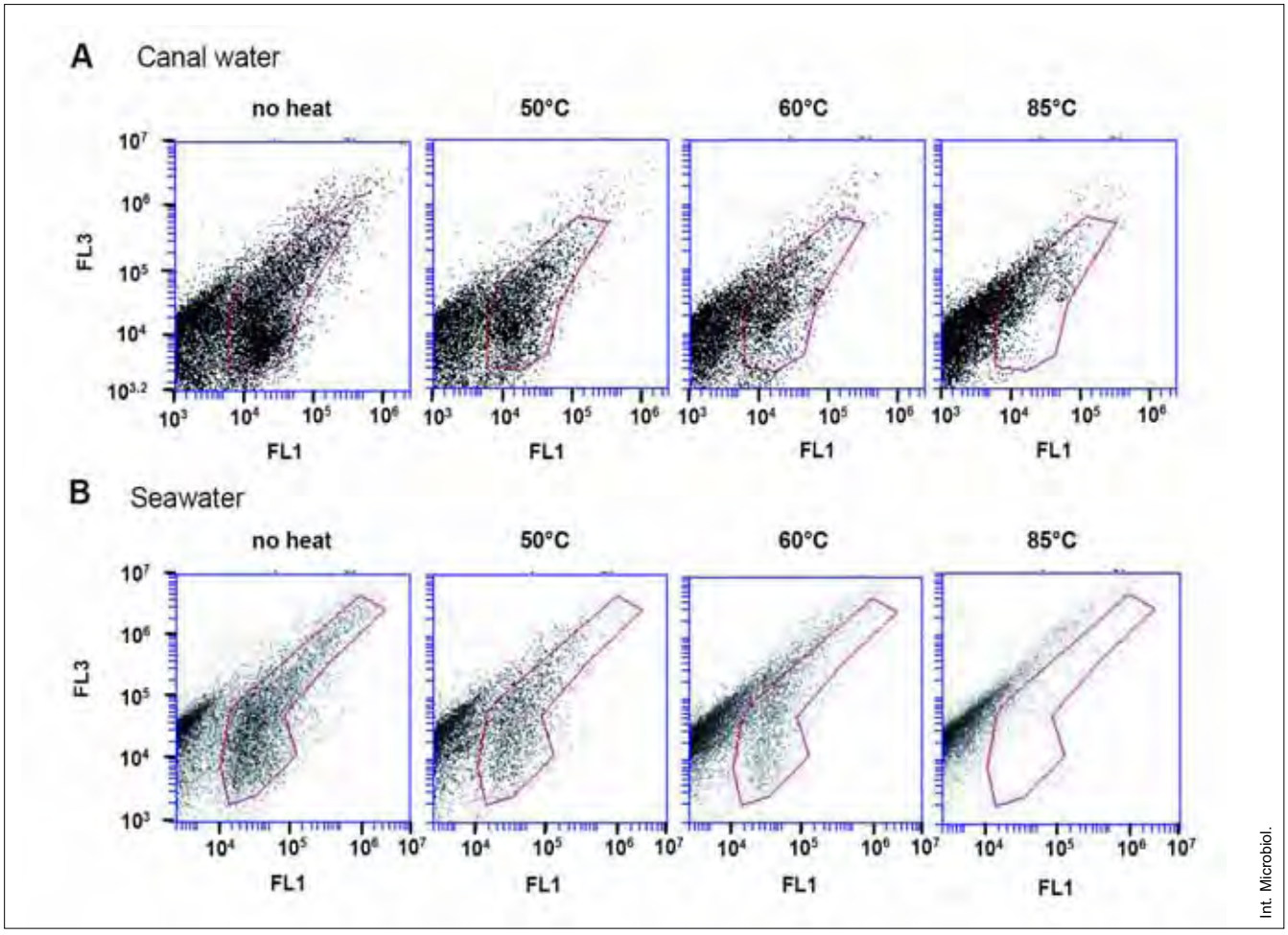

Fig. 2. Flow cytometry analysis of (**A**) canal water and (**B**) seawater samples left untreated or exposed to different temperatures. Samples were stained with the dyes SYTO9 and PI. FL1 denotes the channel detecting green fluorescence signals (530 nm) and FL3 the channel detecting red fluorescence signals (>670 nm). Electronic gates were applied to highlight the regions of most visible change.

brane-compromised cells of one species can, however, be expected in environmental samples subjected to biocidal treatment such as heat exposure.

Exceeding the temperature tolerance of microbial species typically leads to a rapid decline in viability within a narrow temperature range. Temperature tolerance of organisms is expressed as the D-value (time of exposure at which 90% of culturability is lost at a certain temperature). D-values for many waterborne organisms are in the range of minutes at temperatures of 50–60°C [24], although great heterogeneity in D-values can be assumed given the broad diversity of organisms in such environmental samples. Increasing temperatures would have very different effects on species with different temperature tolerances. This was the case in the above-mentioned study for DGGE profiles in a water sediment sample exposed to increasing temperatures [5]: PMA treatment in the presence of rising temperatures resulted in the disappearance of a greater number of bands, which might reflect membrane damage for most cells of those species whose temperature tolerance was exceeded. The same is assumed to hold true for the sequence profiles presented in this study, and a similar outcome is expected for environmental samples treated with disinfectants. In fact, viability often declines rapidly when a certain disinfection strength is exceeded [14].

In our study, PCR amplicons of approx. 280 bp were sequenced. It remains to be tested whether the amplification of longer sequences would contribute to a more efficient exclusion of DNA from membrane-compromised cells. In the case of sample treatment with ethidium monoazide (EMA), the amplification of longer sequences results in a more efficient exclusion of signals from membrane-compromised cells [23]. We have made the same observation in various projects employing PMA (unpublished results). Given the rapid technological advances in 454 sequencing technology and the accompanying longer read lengths, it can be expected that the lengths of amplicons that can be analyzed will be much longer in the future. The amplification of longer sequences would in turn result in a more efficient exclusion of sequences derived from membrane-compromised cells.

In conclusion, we propose the use of PMA treatment in conjunction with 454 microbial sequence profiles under conditions in which samples have been exposed to treatments affecting cell membrane integrity. This approach is likely to be a valuable tool for monitoring the efficiency of sample treatment procedures in which membrane integrity is altered. Although tested only with water samples, PMA treatment might be suitable when applied to other complex communities, with longer amplicon sizes potentially helpful in excluding dead cells more efficiently.

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