

## Brief report

# Application and validation of DNA microarrays for the 16S rRNA-based analysis of marine bacterioplankton

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### Summary

**An oligonucleotide probe-based DNA microarray was evaluated for its ability to detect 16S rRNA targets in marine bacterioplankton samples without prior amplification by polymerase chain reaction (PCR). The results obtained were compared with those of quantitative fluorescence *in situ* hybridization (FISH). For extraction and direct labelling of total RNA, a fast and efficient protocol based on commercially available kits was established. A set of redundant and hierarchically structured probes was applied, and specificity of hybridization was assessed by additional control oligonucleotides comprising single central mismatches. The protocol was initially tested by microarray analysis of bacterial pure cultures. Complete discrimination of all control oligonucleotides was achieved, indicating a high degree of hybridization specificity. In a co-culture, abundant members were detected by microarray analysis, but signal ratios of positive probes did not correlate well with quantitative data from FISH experiments. A marine picoplankton sample from the German Bight was analysed. Bacterial populations with relative abundances of at least 5% were detected by hybridizing 0.1 µg of total RNA extracted from a sample of 375 ml equivalent to  $4.1 \times 10^8$  cells. Our results demonstrate that major populations of marine bacterioplankton can be identified by microarray analysis in a fast and reliable way, even in relatively low volumes of sea water.**

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With the introduction of molecular methods based on comparative analysis of 16S rRNA gene sequences in the 1980s (Olsen *et al.*, 1986), environmental microbiology has entered a new age. One of the latest and most powerful tools in molecular biology is DNA microarray technology, which represents a high-throughput format for the parallel application of multiple nucleic acid probes by reverse hybridization. DNA microarrays are now routinely applied in clinical diagnostics, functional genomics and genetic analysis (Lander, 1999). Although they were adapted for the detection and identification of environmental microorganisms by Guschin and co-workers in 1997 (Guschin *et al.*, 1997), various methodical deficiencies, such as laborious protocols for preparation of target molecules and insufficient detection limits, still constrict a broad application of DNA microarrays in the field of microbial community analysis. So far, only a few studies have been published in which microarrays were used for the parallel detection of 16S rRNAs or amplified 16S rRNA genes from the environment (Rudi *et al.*, 2000; Small *et al.*, 2001; Koizumi *et al.*, 2002; Loy *et al.*, 2002; Wilson *et al.*, 2002; El Fantroussi *et al.*, 2003).

One major issue when hybridizing samples of high complexity is the sensitivity of the analysis. RNA extraction yields from environmental samples are often low, hampering the detection of target populations of low abundance (Koizumi *et al.*, 2002). This limitation can be circumvented by the introduction of an initial polymerase chain reaction (PCR) amplification step with, e.g. general 16S rRNA gene-targeted primers. Concomitantly, PCR enables easy labelling of the target molecules. However, PCR-based methods have been shown to fail to correctly reflect community composition (Suzuki and Giovannoni, 1996; Wintzingerode *et al.*, 1997; Marchesi *et al.*, 1998; Schmalenberger *et al.*, 2001). The direct labelling and hybridization of extracted cellular rRNA (Small *et al.*, 2001; El Fantroussi *et al.*, 2003) promise a less distorted view of true community composition.

To assess the reliability of microarray data obtained from complex microbial communities, convenient methods for their validation are needed. Common strategies are based on a PCR-mediated analysis, such as fingerprinting techniques (Koizumi *et al.*, 2002) or gene libraries (Loy *et*

*al.*, 2002; Wilson *et al.*, 2002). Also, quantitative membrane hybridization was applied for data comparison (Koi-zumi *et al.*, 2002). Whereas PCR-based techniques can provide useful information on the nucleotide sequences of the probe target sites, they do not allow for quantification of the groups targeted.

In this study, a fast and simple protocol for the 16S rRNA-based microarray analysis of marine bacterioplankton communities without prior PCR amplification of the target molecules was established, and samples of different complexity were analysed. Microarray data obtained were compared with parallel quantitative fluorescence *in situ* hybridization (FISH) counts to assess them under the aspects of (i) specificity, (ii) sensitivity and (iii) quantification.

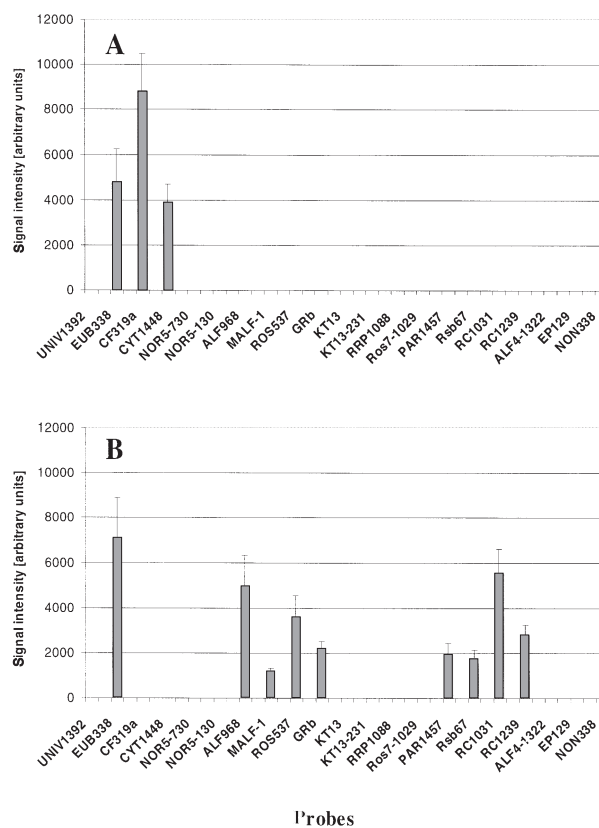
### Extraction and labelling of total RNA from samples of different complexity

For extraction and direct labelling of total RNA from six marine bacterial strains, a co-culture and a marine picoplankton sample, a fast and straightforward protocol based on two commercially available kits was adapted. Only 4 h are needed for sample preprocessing, RNA extraction and labelling, including quality checks. The RNAwiz extraction protocol applied provided intact 16S rRNA from 50 ml of the pure cultures and 100 ml of the co-culture with extraction yields ranging from 30 to 75 µg of total RNA as determined by capillary electrophoresis. These amounts allow for 20–100 hybridization reactions in our microarray format after labelling. Extractions starting from 750 ml of sea water also provided intact RNA, but amounts of total RNA were sufficient for only one or two microarray experiments per preparation. Fractions of the 16S rRNA within the different total RNA preparations varied between 15% and 45% for all types of samples. It is not unusual that the large subunit:small subunit rRNA band intensity ratio differs significantly from the expected 2:1 ratio (Skrypina *et al.*, 2003). Only extractions with a 16S rRNA fraction between 20% and 30% were processed further to guarantee comparability of the data obtained under quantitative aspects. For RNA extraction from the picoplankton sample, a standard protocol according to Oelmüller *et al.* (1990) was also tested, but no differences in total extraction yield, quality or fraction size of the 16S rRNA were observed.

For RNA labelling, the ULYSIS labelling kit was applied, which allows for the direct chemical labelling of nucleic acids by a variety of fluorescent dyes. The relative labelling efficiency of the target molecules was similar for total RNA from the different types of samples. Spectroscopic data suggested one label every 26–41 nucleotides in all extracts analysed.

### Identification of pure cultures by microarray analysis

The suitability of our hybridization format for the direct detection of extracted ribosomal RNA from bacterial cells was determined by initial microarray analysis of labelled RNA from the two environmental strains, KT0202a (alpha-proteobacteria) and KT11ds2 (*Cytophaga-Flavobacterium* group of *Bacteroidetes*), with the redundant and hierarchical set of 16S rRNA-targeted probes shown in Table 1. Additional control oligonucleotides containing single central mismatches were applied to assess hybridization specificity at high resolution. Highly specific signals were observed, and both targets could be clearly differentiated by the signal patterns obtained. Strain KT11ds2 shows three out of four expected signals (one false-negative result for probe UNIV1392) (Fig. 1A), and strain KT0202a shows nine out of 11 expected signals (two false-negative results for probes UNIV1392 and RRP1088) (Fig. 1B). False-negative signals are a common phenomenon in microbial microarray analysis (Koi-zumi *et al.*, 2002; Loy *et al.*, 2002) and can be explained by parameters such as target molecule secondary struc-



**Fig. 1.** Signal patterns obtained by microarray hybridization of extracted total RNAs from strains KT11ds2 (A) and KT0202a (B) to the complete set of probes shown in Table 1 for 3 h with a target concentration of 8 ng µl<sup>-1</sup>. For clarity, single mismatch controls are not shown (here, no unspecific signals occurred).

**Table 1.** Oligonucleotide probes used in this study and their specificities<sup>a</sup>.

Probe	Specificity	Sequence (5'-3')	16S rRNA binding site <sup>b</sup> and length (nt)	GC content (%)	No. of mismatches to target <sup>c</sup>										Reference
					KT0202a	KT1117	JP7.1	JP13.1	KT11ds2	KT71					
UNIV1392	Universal	ACGGCGGTGTGTAC	1392 (15)	67	PM	PM	PM	PM	PM	PM	PM	PM	PM	Pace <i>et al.</i> (1986)	
EUB338	Bacteria	GCTGCCCTCCCGTAGGAGT	338 (18)	67	PM	PM	PM	PM	PM	PM	PM	PM	PM	Amann <i>et al.</i> (1995)	
CF319a	<i>Cytophaga-Flavobacterium</i> group of <i>Bacteroidetes</i>	TGGTCCGTGTCTCAGTAC	319 (18)	56	2 (4, 17)	2 (4, 17)	2 (4, 17)	2 (4, 17)	2 (4, 17)	2 (4, 17)	2 (4, 17)	2 (4, 17)	3	Manz <i>et al.</i> (1996)	
CYT1448	NOR5 cluster of gamma-proteobacteria	CTAGGCCGCTCCTACGG	1448 (18)	67	>4	>4	>4	>4	>4	PM	>4	PM	>4	Eilers <i>et al.</i> (2001)	
NOR5-730		TCGAGCCAGGAGGCCGCC	730 (18)	78	3	3	2 (10, 12)	>4	>4	>4	PM	>4	PM	Eilers <i>et al.</i> (2001)	
NOR5-130	KT71	CCCCACTACTGGATAGAT	130 (18)	50	>4	>4	>4	>4	>4	>4	PM	>4	PM	Eilers <i>et al.</i> (2001)	
ALF968	Alpha-proteobacteria	GGTAAGGTTCTGCGCGTT	968 (18)	56	PM	PM	PM	PM	3	3	PM	1 (12)	1	Neef (1997)	
MALF-1	Marine alpha cluster	GCCGGGGTTTCTTTACCA	488 (18)	56	PM	PM	PM	PM	>4	>4	>4	>4	>4	Gonzalez <i>et al.</i> (1996)	
ROS537	Marine alpha cluster	CAACGCTAACCCCTCC	537 (17)	65	PM	PM	PM	2 (9, 11)	4	4	4	4	4	Eilers <i>et al.</i> (2001)	
GRb	Marine alpha cluster	GTCAGATCGAGCCAGTGAG	735 (20)	55	PM	PM	1 (18)	>4	>4	>4	>4	>4	>4	Giuliano <i>et al.</i> (1999)	
RSB67	Subgroup of marine alpha cluster	CGCTCCACCCGAAGGTAG	67 (18)	67	PM	>4	>4	>4	>4	>4	>4	>4	>4	Zubkov <i>et al.</i> (2001)	
RC1031	KT0202a	ACCTGTCACATATGTCCTCCG	1031 (18)	56	PM	>4	4	2 (12, 18)	>4	>4	>4	4	4	Eilers <i>et al.</i> (2001)	
RC1239	KT0202a	TAACTCACTGTAGTTGCCAT	1239 (20)	40	PM	1 (14)	1 (14)	4	>4	>4	>4	>4	>4	Eilers <i>et al.</i> (2001)	
KT13	Subgroup of marine alpha cluster	TAACTCACTGTAGATGCCAT	1239 (20)	40	1 (14)	PM	PM	3	>4	>4	>4	>4	>4	Peplies <i>et al.</i> (2003)	
KT13-231	KT1117	ATCTAATCAAACGCGGGCC	231 (19)	53	1 (9)	PM	1 (9)	1 (9)	>4	>4	>4	4	4	Eilers <i>et al.</i> (2001)	
Ros7-1029	JP7.1	CTGTACATTTGGTCTCTTG	1029 (18)	50	>4	>4	PM	PM	>4	>4	>4	>4	>4	Eilers <i>et al.</i> (2001)	
RRP1088	Subgroup of alpha-proteobacteria	CGTTGCCGGACTTAACC	1088 (17)	59	PM	PM	PM	1 (7)	3	3	2 (5, 7)	2 (5, 7)	2 (5, 7)	Neef (1997)	
PARI457	Subgroup of alpha-proteobacteria	CTACCGTGGTCCGCTGCC	1457 (18)	72	PM	3	1 (2)	3	>4	>4	>4	>4	>4	Neef (1997)	
ALF4-1322	Subgroup of alpha-proteobacteria	TCCGCCCTCATGCTCTCG	1322 (18)	61	4	4	4	PM	>4	>4	>4	>4	>4	Neef (1997)	
EP129	Subgroup of alpha-proteobacteria	CGAAACCTAAAAGGCAGGTT	129 (18)	50	>4	>4	>4	PM	>4	>4	>4	>4	>4	Neef (1997)	

**a.** For every probe, a control containing one central mismatch was spotted additionally, i.e. the whole probe set includes a total of 40 different oligonucleotides.

**b.** *E. coli* numbering according to Brosius *et al.* (1981); position of 3' nucleotide of probe is stated. nt, nucleotides.

**c.** For up to two mismatches, the position of the mismatches is also stated (numbers in parentheses counted from the 3' end). PM, perfect match.

tures or steric hindrance, reducing hybridization efficiency in a probe binding site-specific way (Peplies *et al.*, 2003). Among 36 potential unspecific hybridization events for strain KT11ds2 and 29 for strain KT0202a, no false-positive signals could be observed including all single mismatch controls. When comparing the results with the corresponding data sets of a previous study in which PCR-amplified 16S rRNA genes of these two strains were analysed (Peplies *et al.*, 2003), nearly identical signal patterns were found. An additional DNA removal step after RNA extraction showed no effect on signal composition and strength (data not shown). Variability of microarray analysis was evaluated for independent hybridizations of identical batches of labelled RNA as well as for independent RNA extractions, and variations in a range of 1–70% (mean 24%) were observed for absolute intensities (arbitrary units) of tested positive signals.

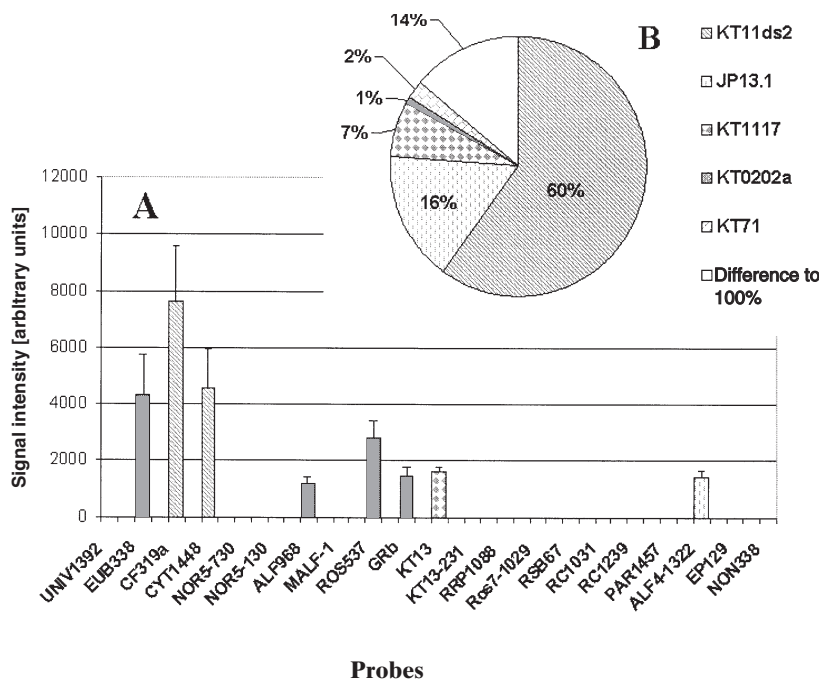
Compared with other protocols used for the direct detection of extracted 16S rRNA on microarrays (Small *et al.*, 2001; Koizumi *et al.*, 2002), no fragmentation of the target molecules was done. Nevertheless, clear hybridization signals were obtained. In terms of sensitivity, the decreased hybridization efficiency of intact RNA (Small *et al.*, 2001) is likely to be counterbalanced by the higher number of reporter groups on each captured target molecule.

For microarray hybridization and washing, a previously optimized protocol (Peplies *et al.*, 2003) was applied with a hands-on time of only 30 min and a hybridization of 3–18 h, depending on the amount of RNA available. This

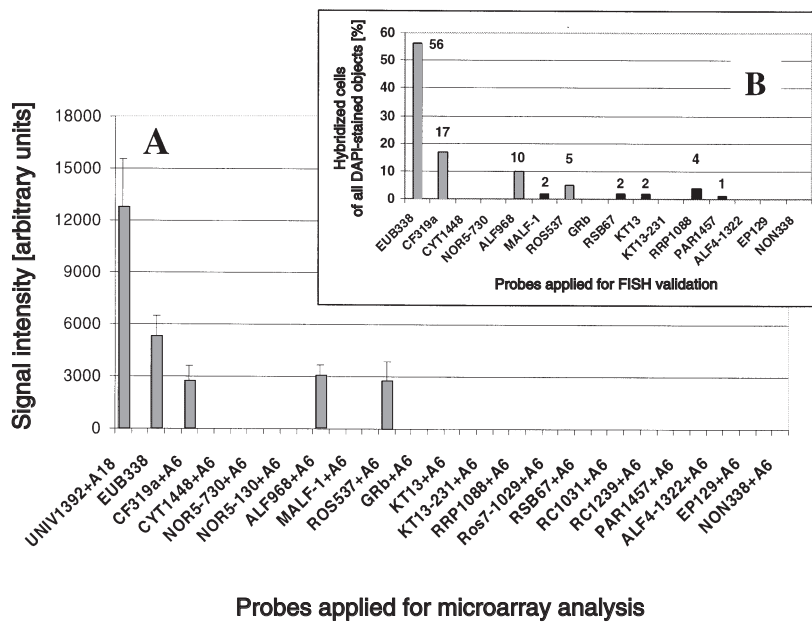
leads to an overall time requirement of 7.5–22.5 h for our protocol. In comparison, Loy *et al.* (2002) specified a total time requirement of 48 h from sampling to data analysis.

### Microarray analysis of bacterial communities and FISH validation

A co-culture of six environmental strains was set up and sampled after 1 week of incubation. The signal pattern obtained after microarray hybridization of 0.45 µg of total RNA (15 ng µl<sup>-1</sup>) from the culture for 3 h (Fig. 2A) suggests the presence of strains KT11ds2, KT1117 and/or JP7.1 and JP13.1 because positive signals were only found for the probes EUB338 (targets all six strains), CF319a, CYT1448 (both only targeting strain KT11ds2), ALF968 (all four alpha-proteobacteria), ROS537, GRb (all three members of the marine alpha cluster), KT13 (only strains KT1117 and JP7.1) and ALF4-1322 (only strain JP13.1). This combination excludes strains KT0202a and KT71 and suggests false-negative results for probes UNIV1392, MALF-1, KT13-231, RRP1088 and EP129 (compare Table 1). Absolute signal intensities ranged from 1175 ± 243 (mean ± standard deviation of spot replicates) arbitrary units (a.u.) for probe ALF968 to 7632 ± 1903 a.u. for probe CF319a. Probes EUB338 and ROS537 yielded signals that were significantly above the background according to our criteria, whereas probes UNIV1392 and MALF-1 did not, although targeting exactly the same strains within the pool of reference strains.



**Fig. 2.** A. Microarray analysis of extracted total RNA from a co-culture after 1 week of incubation, originally inoculated with all six reference strains. Hybridization was performed with a target concentration of 15 ng µl<sup>-1</sup> under standard conditions. For clarity, single mismatch controls are not shown (no signals). Bar patterns indicate probe specificity for the reference strains. Filled bars indicate probes targeting more than one of the reference strains (compare Table 1). B. Relative cellular abundances for the reference strains detected by FISH analysis within the co-culture. Values shown are based on a set of redundant and hierarchically structured probes given in the text. 14% of all DAPI-stained objects showed no positive FISH signals.



**Fig. 3.** A. Microarray analysis of extracted total RNA from the environmental sample. Hybridization was performed for 18 h with a target concentration of  $3 \text{ ng } \mu\text{l}^{-1}$ . For all probes, a 6-mer polyadenosine triphosphate spacer was applied, except for general probes UNIV1302 (A18) and EUB338 (none). For clarity, single mismatch oligonucleotides are not shown (no signals).

B. Parallel FISH analysis of the environmental sample by applying 15 out of the 20 probes selected for microarray analysis (NON338 serves as negative control). The relative cellular abundances of the corresponding target populations are shown. Populations detected by FISH but not by microarray analysis are indicated by black bars.

For validation of the microarray results obtained from the co-culture, probes EUB338, ALF968, MALF-1, RC1031, KT13-231, Ros7-1029, ALF4-1322, CF319a and NOR5-730 were chosen for parallel FISH analysis. The presence of strains KT11ds2, JP13.1 and KT1117 could be confirmed. Only these strains were detected at high relative abundances (60%, 16% and 7% of total DAPI counts respectively) (Fig. 2B). Strains KT71, KT0202a and JP7.1 accounted for 2%, <1% and 0% of the bacterial community respectively. Cell density in the analysed co-culture, as determined by DAPI staining, was  $5.0 \times 10^7 \text{ ml}^{-1}$ . In comparison, Koizumi *et al.* (2002) were able to detect 16S rRNA targets that accounted for at least 16% of total 16S rRNA in a toluene-degrading consortium by microarray hybridization of  $10 \mu\text{g}$  ( $250 \text{ ng } \mu\text{l}^{-1}$ ) of fragmented total community RNA for 16 h. In the study by Small *et al.* (2001), the detection limit for fragmented total RNA from pure cultures added to soil extracts was given with  $0.5 \mu\text{g}$  of the targeted RNAs (representing  $\approx 7.5 \times 10^6$  cell equivalents), based on enzymatic signal amplification. For the two probes NOR5-730 and NOR5-130, targeting only strain KT71 in the co-culture analysed, total RNA isolated from  $\approx 3.0 \times 10^6$  cells gave no positive microarray signals under the conditions tested. Obviously, target concentration was the limiting factor for microarray detection of the less abundant strains KT71 and KT0202a.

For initial microarray analysis of the bacterioplankton sample, hybridization was performed under standard conditions, except that only a reduced amount of RNA was available, resulting in a final concentration of  $3 \text{ ng } \mu\text{l}^{-1}$  ( $0.1 \mu\text{g}$  in total). In this experiment, no signals could be

detected. Therefore, hybridization time was extended to 18 h, and probes were equipped with a 6-mer polyadenosine triphosphate spacer (+A6) (except for the two general probes) to increase sensitivity. Now, microarray analysis gave signals for the general probes UNIV1392 (+A18) and EUB338 and suggested the presence of members of the *Cytophaga-Flavobacterium* group of *Bacteroidetes*, the alpha-proteobacteria and the marine alpha cluster (Fig. 3A). A single microarray hybridization required extraction of a 375 ml subsample. Parallel FISH analysis supported the data obtained by microarray analysis of the bacterioplankton sample (Fig. 3B). The cell density in the analysed sea-water sample was  $1.1 \times 10^6 \text{ cells ml}^{-1}$ . All populations with a relative abundance of at least 5% of total DAPI counts ( $5.5 \times 10^4 \text{ cells ml}^{-1}$ ) were detected by microarray hybridization. Again, no indications for false-positive microarray signals were found. Obviously, a specific hybridization of multiple probes can be assured for complex 16S rRNA target mixtures with our protocol. However, it must be considered that, for FISH, the detection of single mismatch target sites cannot be excluded. On the other hand, signals within the control oligonucleotides would not necessarily indicate unspecific hybridization events because of the high and unknown 16S rRNA sequence diversity within this sample.

So far, only a limited number of studies have compared microarray data with results obtained by independent methods to determine the quantitative composition of microbial communities. Koizumi *et al.* (2002) pointed out that signal ratios of positive probes obtained after microarray analysis do not correlate well with data from quantita-

tive membrane hybridization. In our microarray experiment with the co-culture (Fig. 2A), probes ALF968 and ALF4-1322 yielded signal intensities that were statistically indistinguishable (Student's *t*-test;  $T = 1.967$ ,  $n = 8$ ,  $P = 0.069$ ), although probe ALF4-1322 targets only one (JP13.1) of the two abundant strains targeted by probe ALF968 (JP13.1 and KT1117). As parallel FISH analysis determines cell numbers, it has to be kept in mind that the composition of the extracted community rRNA pool is affected by both cell number and cellular rRNA content of the particular populations (Amann, 1995). However, several probes, applied in parallel and targeting the same 16S rRNA at different sites, also show pronounced variations in signal strength (Figs 1 and 2). Obviously, parameters influencing hybridization efficiency, such as probe sequence, secondary structure of the target molecule and relative position of the probe binding site on the target molecule, interfere with a simple correlation of probe signal ratios and relative abundances of the corresponding target molecules.

In conclusion, a protocol is presented enabling the microarray-based identification of major bacterial populations even in complex and oligotrophic systems such as marine surface waters and, therefore, now allowing for the fast and efficient precharacterization of environmental microbial communities. Further optimizations are needed to increase the sensitivity of the format and to improve the quantification of rRNA populations by microarray hybridization.

## Experimental procedures

### *Bacterial strains, co-culture and environmental samples*

For microarray analysis of extracted RNA from pure cultures and inoculation of a co-culture, six reference strains were used. KT0202a, KT1117, JP7.1, JP13.1 (all alpha-proteobacteria), KT11ds2 (*Cytophaga-Flavobacterium* group of *Bacteroidetes*) and KT71 (gamma-proteobacteria) were originally isolated from the surface water of the German Bight by Eilers *et al.* (2000; 2001). Accession numbers of the 16S rRNA gene sequences of the six strains are AF173971, AF235111, AF305498, AY007676, AY007679 and AY007680.

For setting up a co-culture, 250 ml of the synthetic seawater medium MPM-m (Eilers *et al.*, 2001) supplemented with 0.01% yeast extract was inoculated with all six reference strains using an inoculating loop. Sampling was carried out for parallel microarray and FISH analysis after 1 week of incubation at room temperature on a shaker.

For parallel microarray and FISH analysis of a marine picoplankton sample, surface water was collected on 23 September 2002 at the sampling site Helgoland Roads (54°9'N, 7°52'E) near the island of Helgoland, which is situated 23 miles offshore in the German Bight. After transfer to Bremen (≈10 h), the water was prefiltered (pore size 3 µm) and processed for RNA extraction and FISH analysis.

### *RNA extraction and labelling*

Total RNA was extracted from pure cultures, the co-culture and the environmental sample using the RNAwiz reagent from Ambion, which accords to a classical phenol–chloroform extraction but provides a quick and easy procedure based on a single reagent. Briefly, cells of pure cultures (50 ml) were collected by centrifugation, resuspended in 1 ml of RNAwiz reagent and transferred to a FastRNA Blue tube (Bio101). Cells from the co-culture (100 ml) and the environmental sample (750 ml) were collected on 0.2 µm white polycarbonate membrane filters (Millipore). The filters were cut into small pieces with a sterile razor blade and transferred into 1 ml of the RNAwiz reagent in a Fast RNA Blue tube. For improved cell lysis, tubes were placed into an FP 120 FastPrep cell disruptor (Savant Instruments) and processed at a speed rating of 6 for 2 × 20 s. Further steps were conducted according to the RNAwiz manufacturer's protocol. After the final precipitation step, the RNA was resuspended in 50 µl of PCR water (Sigma). Optionally, potential DNA contaminations of the RNA extracts were removed by the DNA-free kit from Ambion.

Alternatively, total RNA from the environmental sample was extracted based on a hot phenol–chloroform treatment according to Oelmüller *et al.* (1990).

Quality and quantity of the extracted RNA were checked by capillary electrophoresis using an Agilent 2100 bioanalyser with the RNA 6000 Nano assay kit (Agilent Technologies), allowing quantification of rRNA fragments.

Total RNA was labelled using an Alexa Fluor 488 ULYSIS labelling kit from Molecular Probes according to the manufacturer's protocol, except that, for purification, a second precipitation step was applied, and precipitation was conducted at room temperature. This kit provides a rapid (10 min), non-enzymatic method for chemical labelling of nucleic acids without fragmentation based on a platinum dye complex, resulting in stable co-ordination complexes with guanine residues. The labelled RNA was eluted in 25 µl of Tris–EDTA buffer, pH 7.5. Finally, labelling efficiency and quantity of the RNA were determined by UV spectrometry according to the manufacturer's protocol. Labelled RNA was stored at –18°C.

### *Oligonucleotide probe set*

The probes and their characteristics are listed in Table 1. Part of the probes target abundant bacterial groups that can be found in the German Bight surface waters (Eilers *et al.*, 2001). For all 20 probes, a control containing one single central mismatch was included. A probe reverse complementary to EUB338, termed NON338, was spotted additionally (not listed in Table 1) to serve as a general negative control when analysing samples of unknown composition.

### *Preparation of glass slides and spotting*

For covalent immobilization of 5' amino-modified capture oligonucleotides (Thermo Hybaid), standard microscopic glass slides (Menzel) were activated by 1,4-phenylenediisothiocyanate (PDITC) treatment according to Benters *et al.* (2002).

Probes were spotted onto the activated slide surface using a SpotArray24 spotting device with TeleChem Stealth pins (Packard BioChip Technologies). Concentration of the amino-modified oligonucleotides in PCR water was 10  $\mu\text{M}$  with 1% glycerol. Normal spot diameter was  $\approx 100 \mu\text{m}$  under the spotting conditions applied. Post-processing of the spotted slides (binding, blocking, washing and storing) was conducted as described recently (Peplies *et al.*, 2003).

#### DNA microarray hybridization

For hybridization and washing of spotted slides, a standard FISH protocol was used according to Pernthaler *et al.* (2001), as described recently (Peplies *et al.*, 2003). In all cases, hybridization was performed at 46°C without formamide. Standard hybridization time was extended to 3 h compared with the FISH protocol. Total RNA from the picoplankton sample was also hybridized for 18 h. Unless otherwise specified, a total amount of 0.23  $\mu\text{g}$  of extracted and labelled total RNA was applied to the microarrays in a volume of 30  $\mu\text{l}$  of hybridization buffer (final concentration 8  $\text{ng } \mu\text{l}^{-1}$ ) under a 22  $\times$  25 mm LifterSlip (Erie Scientific). Hybridization was conducted in a Corning hybridization chamber. Unless otherwise specified, probes were applied for microarray analysis under standard conditions, i.e. immobilized via the 5' end, without spacer and with a concentration of 10  $\mu\text{M}$ . After washing, slides were dried by centrifugation at low speed. For reduction of steric hindrance, probes were used with polyadenosine triphosphate spacers six nucleotides in length, located at the 5' end of the capture oligonucleotides.

#### Signal detection and data analysis

Slides were imaged at a resolution of 10  $\mu\text{m}$  using a ScanArray Express microarray scanner (Packard BioChip Technologies) at identical sensitivity settings of the photomultiplier (75%) and a laser power of 70%.

For spot detection and signal quantification, the microarray analysis software QUANTARRAY 3.0 (Packard BioChip Technologies) was used. For spot quantification, the fixed circle method was used. Signals were considered as positive if mean spot pixel intensity was higher than mean local background pixel intensity plus twice the standard deviation of the local background. This range decreases the probability of false-positive signals to <2.5%. All probes were deposited in 10 replicate spots. Each data point shown represents the arithmetic mean of the positive replicates for a particular probe. Error bars indicate the standard deviation of the positive replicates.

#### Fluorescence in situ hybridization

Microarray analysis of samples with an unknown community composition was validated by FISH using the standard protocol (Pernthaler *et al.*, 2001). Probes UNIV1392, EUB338, CF319a, CYT1448, NOR5-730, ALF968, MALF-1, ROS537, GRb, KT13, RSB67, KT13-231, RRP1088, PAR1457, ALF4-1322, EP129, RC1031 and Ros7-1029 were used at 0%, 35%, 35%, 30%, 30%, 20%, 20%, 35%, 30%, 20%, 20%, 30%, 0%, 20%, 35%, 0%, 20% and 35% formamide respec-

tively. Briefly, 1 ml of the co-culture and 5 ml of the water sample were fixed with 2% (v/v) formaldehyde for 1 h at 4°C and filtered on 0.2  $\mu\text{m}$  white polycarbonate membrane filters (Millipore). Cells on filter sections were hybridized, counterstained with 4,6-diamidino-2-phenylindole (DAPI), mounted and evaluated microscopically as described previously (Pernthaler *et al.*, 2001).

#### Acknowledgements

We thank D. Wöhrle and B. Meyer-Schlosser for generous supply of activated glass slides. A. Ellrott is gratefully acknowledged for excellent help in setting up and maintaining technical equipment. This work was done in the framework of the Centre of Applied Gensensorik (CAG) at the University of Bremen and was supported by the German Ministry of Education and Research (BMBF, contract 0311833A) and by the Max Planck Society.

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