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mRNA-Based Parallel Detection of Active Methanotroph Populations by Use of a Diagnostic Microarray

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A method was developed for the mRNA-based application of microbial diagnostic microarrays to detect active microbial populations. DNA- and mRNA-based analyses of environmental samples were compared and confirmed via quantitative PCR. Results indicated that mRNA-based microarray analyses may provide additional information on the composition and functioning of microbial communities.

Microarrays are increasingly applied in environmental and medical microbiology for microbial diagnostics (3, 9, 17). Microbial diagnostic microarrays (MDMs) based on functional genes (encoding enzymes involved in a specialized biochemical process, such as methane oxidation), also termed functional gene arrays, focus the investigation onto a selected functional group of bacteria, also enabling the analysis of hitherto uncultivated members of that group. Currently applied target preparation methods, starting from environmental DNA, deliver information about the presence of bacteria but not about their level of physiological activity. Various forms of inactive bacteria (dormant cells, viable but nonculturable cells, spores, and dead cells with DNA not yet degraded) are all detected like active cells. In most cases, information on the active populations would be desirable. DNA- and RNA-based 16S clone libraries were shown to reveal different bacterial populations from the same community (11). Stable isotope probing (10, 12, 13), fluorescent in situ hybridization (2, 6, 16), and isotope arrays (1) are techniques with the potential to deliver information on the active populations.

Methanotrophs are bacteria capable of utilizing methane as their sole source for carbon and energy, representing the largest biological sink for the greenhouse gas methane. Type Ia methanotrophs comprise the mesophilic members of methanotrophs within the γ-*Proteobacteria* (*Methylomonas*, *Methylobacter*, *Methylosarcina*, and *Methylomicrobium*); type Ib consists of the thermotolerant/thermophilic members of the same group (*Methylococcus*, *Methylocaldum*, and "*Methylothermus*"). Type II methanotrophs comprise the methanotrophs within the α-*Proteobacteria* (*Methylosinus*, *Methylocystis*, *Methylocapsa*, and *Methylocella*) (7).

We have developed a method for the mRNA-based application of MDMs to detect active microbial populations. The method was tested using a *pmoA* microarray (4, 15) for methanotrophs and samples from lysimeters simulating open landfill covers with planted compost.

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For environmental mRNA purification, the method of Bürgmann et al. (5) was modified. Glass beads (0.75 g) and 0.5 g of fresh soil were added to 1.25 ml of cold CTAB+ buffer (0.2% [wt/vol] cetyltrimethylammonium bromide [CTAB], 0.2 M sodium phosphate, pH 8.0, 0.1 M sodium chloride, 50 mM EDTA in sterile RNase free water, 1 mM dithiothreitol added fresh before use) in 2.0-ml Eppendorf tubes and were processed in a FastPrep bead beater (Bio 101/Savant, Farmingdale, N.Y.) for 45 s at 6 m s^{-1}. Lysed samples were cooled on ice for 1 min and centrifuged for 5 min at 10,000 relative centrifugal force (RCF). Supernatants were subjected to routine phenol-chloroform-isoamyl alcohol extraction (14). Purified supernatants (800 μl) were mixed with 400 μl of 7.5 M potassium acetate, chilled on ice for 1 h, and then centrifuged (10,000 RCF for 5 min at 4°C). The aqueous phase was recovered and subjected to routine chloroform-isoamyl alcohol extraction (14); 700 μl of supernatant was transferred to fresh 1.5-ml tubes, and 750 μl of polyethylene glycol precipitation solution (20% [wt/vol] polyethylene glycol 6000, 2.5 M NaCl in sterile RNase free water) was added. After brief vortexing, samples were incubated for 1 h at 37°C and then centrifuged for 30 min at 10,000 RCF at 37°C. Pellets at this stage were light brown.

For RNA cleanup, pellets were air dried at room temperature (15 min) and resuspended in 35 μl of an aqueous mixture containing 3.5 μl of 10× DNase I reaction buffer and 3 μl of 1U/μl DNase I (Promega, Madison, Wis.). Resuspended nucleic acids were incubated at 25°C for 15 min and then put on ice to stop the reaction. Sterile water (15 μl) and 125 μl of CRSR buffer (0.6% [wt/vol] CTAB, 50 mM sodium-acetate, pH 4.5, in sterile RNase-free water, 1 mM dithiothreitol added fresh before use) was added and mixed. Routine phenol-chloroform-isoamyl alcohol extraction (14) was performed, followed by chloroform-isoamyl alcohol extraction (14). The supernatant (145 μl) was transferred to a fresh 0.5-ml Eppendorf tube, and 1 μl of 10 mg ml^{-1} glycogen solution and 200 μl of isopropanol were added and incubated at room temperature for 3 min and then on ice for another 3 min. RNA was pelleted by centrifugation at 4°C for 10 min at 10,000 RCF. The RNA pellet was washed with 500 μl of ice-cold ethanol and centrifuged for 5 min at 10,000 RCF (4°C). The pellet was air dried

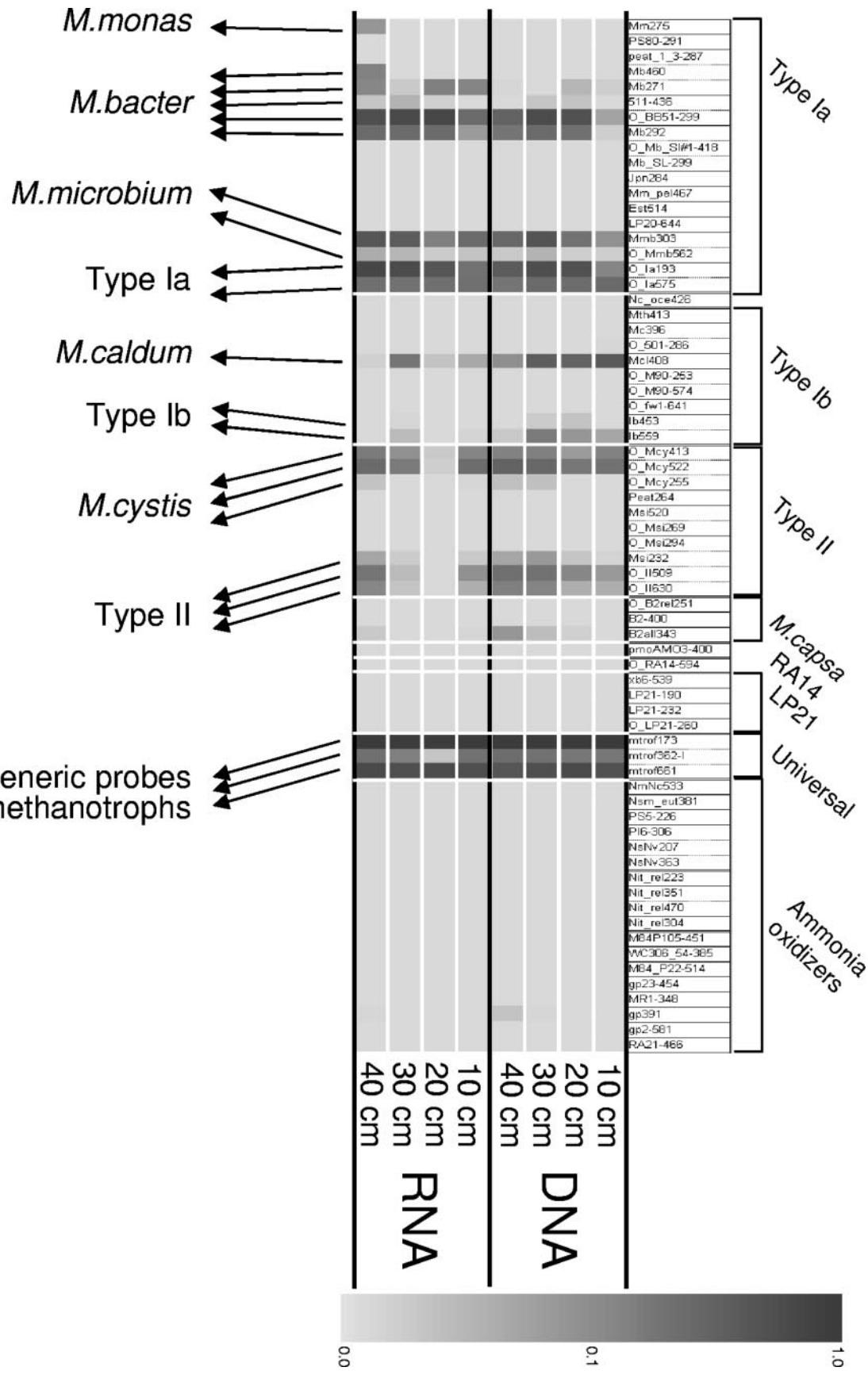
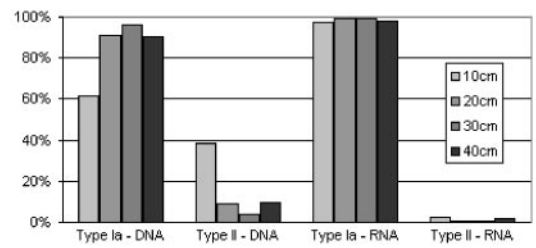


FIG. 1. Methanotroph community analyses using environmental DNA and mRNA. Results of individual microarray experiments were first normalized to positive control probe mtrof173 and then to the reference values determined individually for each probe (see reference 15) and displayed using GeneSpring software. In essence, a value of 1.0 indicates maximum achievable signal for an individual probe, while a value of 0.1 indicates that about 10% of the total PCR product hybridized to that probe. Color coding is indicated on the side bar. *M.monas*, *Methylomonas*; *M.bacter*, *Methylobacter*; *M.microbium*, *Methylomicrobium*; *M.caldum*, *Methylocaldum*; *M.cystis*, *Methylocystis*; *M.capsa*, *Methylocapsa*.

A

	DNA		RNA	
	Type Ia - DNA	Type II - DNA	Type Ia - RNA	Type II - RNA
10cm	3,99E+06	2,52E+06	2,31E+06	6,01E+04
20cm	2,18E+07	2,18E+06	1,29E+07	6,24E+04
30cm	6,48E+07	2,80E+06	3,66E+07	3,46E+05
40cm	1,63E+08	1,80E+07	5,57E+07	9,39E+05

B



	Type Ia - DNA	Type II - DNA	Type Ia - RNA	Type II - RNA
10cm	61%	39%	97%	3%
20cm	91%	9%	100%	0%
30cm	96%	4%	99%	1%
40cm	90%	10%	98%	2%

FIG. 2. Quantitative PCR results. (A) Number of target DNA/mRNA molecules per gram of soil. (B) Relative abundance of type Ia and type II target molecules expressed as a percentage of the sum of type Ia and type II molecules.

at room temperature and resuspended in ice-cold RNase-free water to the desired volume. The absence of DNA contamination was confirmed by carrying out PCR amplification of *pmoA* as described below.

For DNA cleanup, nucleic acid pellets from the Burgmann method were air dried at room temperature (15 min) and resuspended in 50 μl of sterile water containing 50 μg/ml RNase A, incubated at 25°C for 15 min, and then put on ice to stop the reaction. For final purification, spin columns that contained Sepharose CL-6B (Pharmacia) and 20 mg ml^{-1} polyvinylpyrrolidone (Sigma) were used.

Target generation for the microarray work was performed by PCR amplification of the *pmoA* gene from DNA as described previously, using modified primers based on the pmoA189-mb661 primer pair (15). The modification involved the addition of a T7 promoter site tag to the 5′ end of the mb661 primer, allowing for a T7 RNA polymerase-based reverse transcription (RT) of the PCR products. For target generation from RNA, 200 to 250 ng of environmental RNA was reverse transcribed using a SuperScript I reverse transcriptase kit (Invitrogen Corp., Carlsbad, Calif.) following the manufacturer's recommendations, using random hexamers as primers. The resulting cDNA was used for PCR amplification according to the same method used for environmental DNA. Fluorescent target generation via in vitro transcription and microarray hybridization was carried out as described earlier (15).

DNA- and mRNA-based microarray analysis of the depth profile of a lysimeter showed marked differences (Fig. 1). *Methylocaldum* populations (detected by probe Mcl408 and broad specificity type Ib-specific probe Ib559) were found to be much more dominant throughout the depth profile with DNA-based analysis than with mRNA-based analysis, indicating the presence of a low-activity population. Type II *Methylocystis* was found to be present throughout the soil core (probes Mcy413 and Mcy522 as well as universal type II probes Msi232, II509, and II630) with DNA but not in the 20-cm layer when RNA was analyzed. The fact that no type II methanotrophs were detected in this sample has to be considered with the relative detection limit (of around 5% of the analyzed microbial community) of the technique. Quantitative RT-PCR data indicated that the ratio of type II methanotrophs was by far the lowest in this sample (it also has to be noted that in this experiment there was only one replicate per sample analyzed, leaving the possibility that the lack of *Methylocystis* in this layer is an artifact of the RNA preparation). Finally, some type Ia methanotrophs were found only with the RNA approach (*Methylomonas*, probe Mm275, and two subgroups of the *Methylobacter* genus, probes Mb460 and Mb271). Considering that the current detection threshold for the methanotroph microarray is 5% of the *pmoA* genes in the PCR amplification (15), the latter results indicate that this *Methylomonas* group and the two *Methylobacter* groups were low in abundance (each representing less than 5% of the methanotrophs targeted) but were exhibiting relatively high activity.

Quantitative PCR and quantitative RT-PCR were carried out to support microarray results, based on the method described by Kolb et al. (8). In short, 10 ng of environmental DNA or cDNA was used as a template for a three-step thermoprofile: denaturation (94°C for 25 s), annealing (the annealing temperature for the primers was used; 20 s) and elongation (72°C for 45 s). The pmoA189/Mb601R and II223/II646 primer pairs were used for the quantification of type Ia and type II methanotrophs at the annealing temperature of 50 and 58°C, respectively. Detection was done at the annealing temperatures. Melting curve analysis indicated the absence of primer dimers at these temperatures. Results were normalized against quantitative PCR runs with calibration standards of *Methylomonas methanica* S1 and *Methylocystis parvus* OBBP *pmoA* PCR products with known numbers of target molecules. Quantitative PCR and quantitative RT-PCR results were in good agreement with the microarray results, as indicated by the similar ratios between type Ia and type II methanotrophs (Fig. 2).

We analyzed the *pmoA* gene transcripts from a larger-scale lysimeter experiment using the microarray approach. The lysimeter experiment was performed in triplicate with alfalfa as the plant cover as described previously (15). In the previous experiment, the *pmoA* gene was detected using DNA as the target. In this study, the mRNA from the lysimeters (extracted at the same time as the DNA) was analyzed. A broad diversity of type Ia methanotrophs was detected using the mRNA approach (*Methylomonas*, *Methylomicrobium*, and two subgroups of the *Methylobacter* genus), none of which had been detectable when the microarray analysis had been done from DNA (Fig. 3). These results indicate that very different information can be obtained when gene transcripts, rather than the genes themselves, are detected.

We have shown that mRNA-based MDM analyses may provide additional information on the composition and functioning of a microbial community. The detection and relative signal

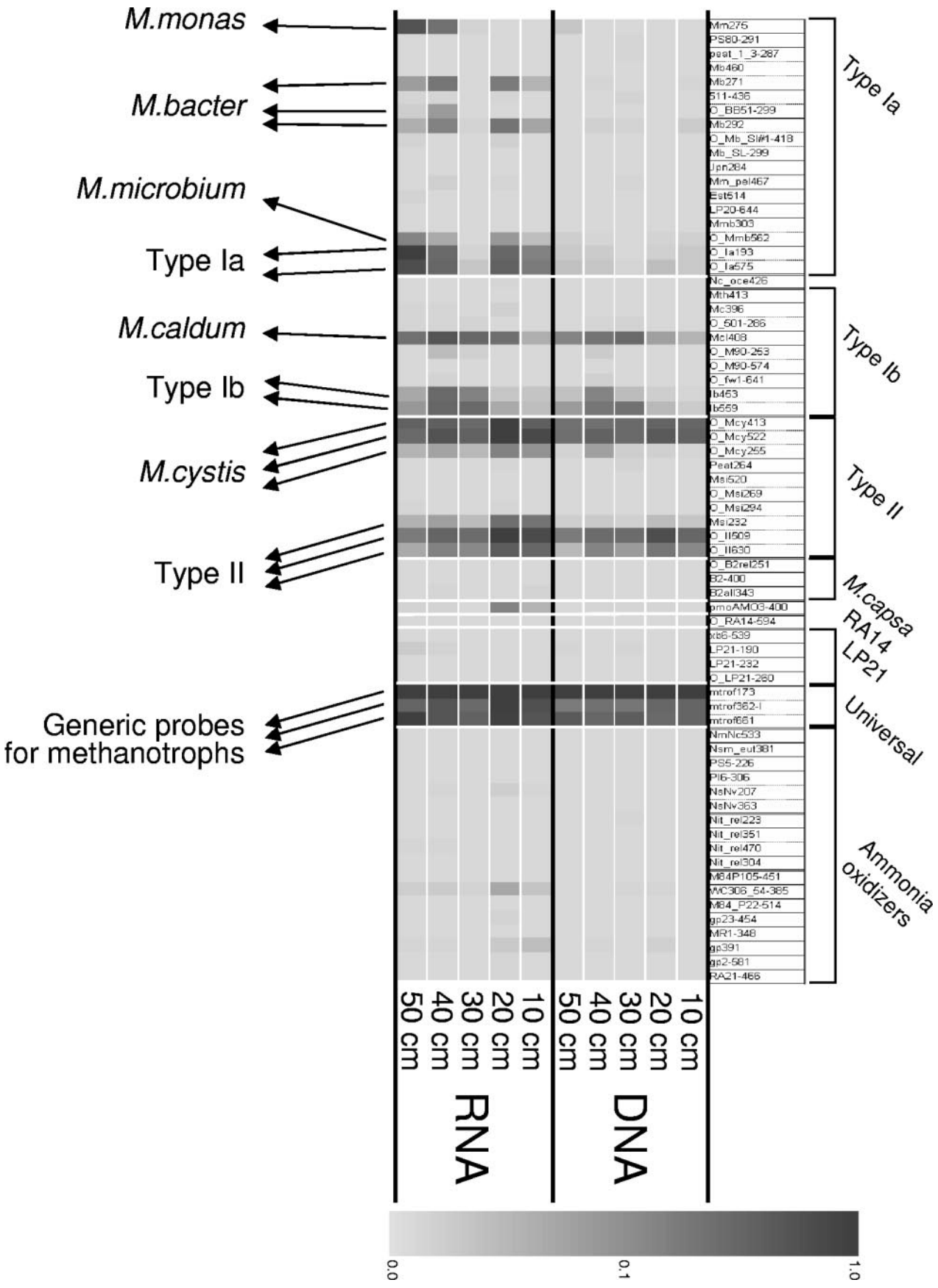


FIG. 3. Methanotroph community analyses using environmental DNA and mRNA. Results of individual microarray experiments were first normalized to positive control probe mtrof173 and then to the reference values determined individually for each probe (see reference 15), averaged between parallels (three parallel lysimeters), and displayed using GeneSpring software. In essence, a value of 1.0 indicates maximum achievable signal for an individual probe, while a value of 0.1 indicates that about 10% of the total PCR product hybridized to that probe. Color coding is indicated on the side bar. *M.monas*, *Methylomonas*; *M.bacter*, *Methylobacter*; *M.microbium*, *Methylomicrobium*; *M.caldum*, *Methylocaldum*; *M.cystis*, *Methylocystis*; *M.capsa*, *Methylocapsa*.

for a detected bacterial group are due to two factors: (i) the abundance and (ii) the mRNA level of that group, the latter of which is related to activity levels. Due to the short lifetime of mRNAs, expression analysis yields information on bacterial activities at the time point of sampling, and transcript levels may be highly sensitive to altering environmental conditions. Therefore, the information obtainable with the present technique needs to be treated with care. DNA-based community analysis results are expected to be more stable but suffer from the limitation that cells irrespective of their activity are detected. Functional MDMs have the potential to be used for high-throughput analysis of bacterial gene expression in natural environments over time and across space, which will lead to a better understanding of the various factors that influence functional activities.

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