Environmental Microbiology (2004) **6**(4), 347–363 doi:10.1111/j.1462-2920.2004.00582.x

Optimization of diagnostic microarray for application in analysing landfill methanotroph communities under different plant covers

Nancy Stralis-Pavese,[1] Angela Sessitsch,[1] Alexandra Weilharter,[1] Thomas Reichenauer,[2] Johann Riesing,[2] József Csontos,[3] J. Colin Murrell[4] and Levente Bodrossy[1]*

[1]*Department of Biotechnology, ARC Seibersdorf research GmbH, A-2444 Seibersdorf, Austria.*
[2]*Department of Environmental Research, ARC Seibersdorf research GmbH, A-2444 Seibersdorf, Austria.*
[3]*Institute of Biotechnology, Bay Zoltán Applied Research Institute, Szeged, Hungary.*
[4]*Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, UK.*

Summary

Landfill sites are responsible for 6–12% of global methane emission. Methanotrophs play a very important role in decreasing landfill site methane emissions. We investigated the methane oxidation capacity and methanotroph diversity in lysimeters simulating landfill sites with different plant vegetations. Methane oxidation rates were 35 g methane m^{-2} day^{-1} or higher for planted lysimeters and 18 g methane m^{-2} day^{-1} or less for bare soil controls. Best methane oxidation, as displayed by gas depth profiles, was found under a vegetation of grass and alfalfa. Methanotroph communities were analysed at high throughput and resolution using a microbial diagnostic microarray targeting the particulate methane monooxygenase (*pmoA*) gene of methanotrophs and functionally related bacteria. Members of the genera *Methylocystis* and *Methylocaldum* were found to be the dominant members in landfill site simulating lysimeters. Soil bacterial communities in biogas free control lysimeters, which were less abundant in methanotrophs, were dominated by *Methylocaldum*. Type Ia methanotrophs were found only in the top layers of bare soil lysimeters with relatively high oxygen and low methane concentrations. A competetive advantage of type II methanotrophs over type Ia methanotrophs was indicated under all plant covers investigated. Analysis of average and individual results from parallel samples was used to identify general trends and variations in methanotroph community structures in relation to depth, methane supply and plant cover. The applicability of the technology for the detection of environmental perturbations was proven by an erroneous result, where an unexpected community composition detected with the microarray indicated a potential gas leakage in the lysimeter being investigated.

Received 7 October, 2003; accepted 9 December, 2003. *For correspondence. E-mail levente.bodrossy@arcs.ac.at; Tel. (+43) (0) 50550 3548; Fax (+43) (0) 50550 3444.

Introduction

Methane is an important greenhouse gas with a current atmospheric concentration of 1.7 p.p.m. by volume, an average increase rate of 6% for 1990–2000, and a global warming potential of 23 (relative to that of carbon dioxide) (International Panel on Climate Change (IPCC); http://www.ipcc.ch). Methane contributes approximately 15% to the total anthropogenic greenhouse effect (Rodhe, 1990). Landfill sites are estimated to release 36–73 Tg of methane annually (Lelieveld *et al*., 1998; Howeling *et al*., 1999; Olivier *et al*., 1999) which is 6–12% of global methane emissions (estimated at 598 Tg; IPCC; http://www.ipcc.ch). Reeburgh (1996) estimated global methane production by landfills at 62 Tg per year with 22 Tg being oxidized by methanotrophs and 40 Tg emitted. Methane removal rates reported for different landfill sites vary between 10 and 100% (Hilger and Humer, 2003). Methanotrophs are a physiologically distinct group of bacteria utilizing methane as their sole source of carbon and energy. They are ubiquitous in nature and represent the largest biogenic sink for the greenhouse gas methane. They oxidize methane via methanol and formaldehyde to carbon dioxide or incorporate carbon from methane into cell biomass at the oxidation level of formaldehyde. The first step in the pathway is catalysed by one of the two types of the enzyme methane monooxygenase (MMO). The soluble, cytoplasmic MMO (sMMO) is found in only some of these bacteria whereas the particulate, membrane bound MMO (pMMO) is present in all known methanotrophs (Hanson and Hanson, 1996) except for *Methylocella palustris* (Dedysh *et al*., 2000). The sequence of *pmoA* encoding the 27 kDa subunit of pMMO

reflects evolutionary relationships amongst *pmoA* containing bacteria (Holmes *et al*., 1995). The ammonia monooxygenase (AMO) of autotrophic ammonia oxidizing bacteria (AOB) is evolutionarily related to pMMO and *pmoA* has a high degree of identity with *amoA*, the gene encoding for the corresponding subunit of the AMO (McDonald and Murrell, 1997). Both *pmoA* and *amoA* genes can be present in one to three, generally highly similar copies in the genomes of methane and ammonia oxidizers (Mctavish *et al*., 1993; Semrau *et al*., 1995; Purkhold *et al*., 2000; Bourne *et al*., 2001; Stolyar *et al*., 2001). There are genes cloned from environmental samples, distantly related to *pmoA*/*amoA*, where it is not clear whether the encoded enzyme is oxidizing methane and/or ammonia, or neither of them (Holmes *et al*., 1999; Henckel *et al*., 2000a; Bourne *et al*., 2001).

Methanotrophs are traditionally classified into two taxonomical groups, type I and type II, based on their 16S rRNA phylogeny, carbon assimilation pathways, PLFA profiles and the architecture of their intracellular membrane system (Hanson and Hanson, 1996). Type I (the family *Methylococcaceae,* Bowman, 1999) is composed of the genera *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylosarcina*, *Methylosphaera*, *Methylococcus*, *Methylocaldum* and *'Methylothermus'* (Bodrossy *et al*., 1997; 1999; Bowman *et al*., 1997; Wise *et al*., 2001). Type II (the family *Methylocystaceae*, Bowman, 1999) includes the genera *Methylosinus* and *Methylocystis*. Two recently described genera, *Methylocapsa* and *Methylocella* form a tight taxonomic group with *Beijerinckia indica* and are related to type II methanotrophs, based on their 16S rRNA and *pmoA* sequences (Dedysh *et al*., 2000; 2002). Also recently, novel psychrophilic methanotrophs were isolated from Canadian tundra samples with *pmoA* sequences grouping together with *amoA* sequences of ammonia oxidizers (Pacheco-Oliver *et al*., 2002).

Mesophilic, neutrophilic type I (type Ia; *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylosarcina*, *Methylosphaera*) and type II methanotrophs co-exist in many environments. The most important factor in their competition are methane and oxygen partial pressures and the availability of fixed nitrogen. Type Ia methanotrophs appear to outcompete type II strains under oxygen-rich, methane limited conditions (Amaral and Knowles, 1995; Henckel *et al*., 2000b). Most type II methanotrophs and some *Methylomonas* strains (type Ia) are capable of nitrogen fixation, thus having a competitive advantage under nitrogen limited conditions (Murrell and Dalton, 1983; Auman *et al*., 2001). Type II methanotrophs form exospores conferring some resistance to them against fluctuations in nutrient supply (Vecherskaya *et al*., 1993).

Landfill site cover soils have the highest (aerobic) methane oxidation capacity reported for any environments; it is 1–2 orders of magnitude higher than that of other aerobic environments (Whalen *et al*., 1990; Kightley *et al*., 1995; Borjesson *et al*., 1998). Considering the large amounts of methane produced in landfill sites our understanding of the community structure of methanotrophs is crucial for the control of the greenhouse effect. The design of better landfill management practices requires a detailed understanding of impacts increasing or decreasing the methane oxidation potential of landfill cover soils. A mildly acidic landfill cover soil was shown to contain members of the genera *Methylocystis*, *Methylosinus* and *Methylosarcina* (Wise *et al*., 1999; 2001). Both type I and type II methanotrophs were detected by PLFA profiling in Swedish landfill sites (Borjesson *et al*., 1998). It has also been noted that exopolysaccharides produced by methanotrophs can form a white layer in landfill cover soils at varying depths of 5–45 cm, potentially limiting the diffusion of oxygen into deeper layers (Hilger *et al*., 2000; Chiemchaisri *et al*., 2001).

Microbial diagnostic microarrays contain sets of oligonucleotide probes targeting different strains, species, genera and higher taxonomic groups of microbes. They offer the potential for the rapid, high-resolution analysis of mixed microbial populations from a large number of samples. Whereas the technological development for such microarrays is very intense, there are very few publications describing the full-scale application of these microarrays (Chandler, 2002; Koizumi *et al*., 2002; Loy *et al*., 2002; Rudi *et al*., 2002; Wilson *et al*., 2002; Peplies *et al*., 2003).

We have developed a microbial diagnostic microarray for the detection and community analysis of methanotrophic bacteria (Bodrossy *et al*., 2003). The microarray consists of oligonucleotide probes designed against the *pmoA*/*amoA* genes of methanotrophs and related bacteria.

In the present work we applied our methanotroph diagnostic microarray to the analysis of methanotroph communities in lysimeters simulating landfill site cover soils with different vegetations. The aim of our work was twofold. First, we wanted to investigate the effect of plant cover on methanotroph communities in landfill site cover soils and to relate these to differences in methane oxidation potential of the cover soils. Second, we wanted to demonstrate the potential of diagnostic microbial microarrays in environmental microbiology.

Results and discussion

Probe set improvement

The final probe set consisted of 68 oligonucleotide probes targeting the *pmo*A/*amo*A genes of methanotrophs, ammonia oxidizers and functionally related bacteria (Table 1). Compared with our previous probe set (Bodrossy *et al*., 2003), several improved and novel probes were designed and validated.

Table 1. Final oligonucleotide probe set. Mismatch sites are indicated by boldfaced and underlined characters. Melting temperatures were calculated by the nearest neighbour method. *M. bacter* = *Methylobacter*, *M. monas* = *Methylomonas*, *M. microbium* = *Methylomicrobium*, *M. sarcina* = *Methylosarcina*, *M. coccus* = *Methylococcus*, *M. thermus* = *Methylothermus*, *M. caldum* = *Methylocaldum*

Name[a]	Intended specificity	Sequence 5′ → 3′[b]	L	T_m	New[c]	MM[d]
Mm275	*Methylomonas*	GTGGTGGAGATACCGTTTGCC	21	59.2	–	A A
PS80-291	clone PS-80	ACCAATAGGCGCAACACTTAGT	22	58.3	–	
peat1-3-287	*Mehtylomonas*-related peat clones	AACTGCCTTTAGGCGCTACC	20	58.6	–	
Mb460	*Methylobacter*	GACAGTTACAGCGGTAATCGGTGG	24	60.9	–	
Mb271	*Methylobacter*	TTGTGGTGGCGTTACCGT	18	58.0	–	C T C
511-436	*Methylobacter*	GTTTTGATGCTGTCTGGCAG	20	55.5	–	ACC AA
BB51-302	*Methylobacter*	CGGTTGTTTGTGTCTTAGGTCTG	23	57.2	New	
Mb292	*Methylobacter*	CCGTTACCGTCTGCCTTTCG	20	59.1	–	G/T T AT
Mb_SL#1-418	soda lake *Methylobacter* isolates and clones	GCGATCGTATTAGACGTTATCCTGATG	27	58.6	New	
Mb_SL-299	soda lake *Methylobacter* isolates and clones	GGGGTGCAACTCTGTGTATCTTAGG	25	60.5	–	T
Jpn284	clone Jpn 07061	ACCGTATCGCATGGGGTG	18	58.0	–	
Mm_pel467	*Methylomicrobium pelagicum*	ACTGCGGTAATCGATGGTTTGGC	23	61.6	–	
Est514	*Methylomicrobium*-related clones	AATTGGCCTATGGTTGCGCC	20	59.9	–	
LP20-644	*Methylomicrobium*-related clones	GGTACACTGCGTACTTTCGGTAA	22	56.0	–	
Mmb303	*Methylomicrobium album*	CAATGCTGGCTGTTCTGGGC	20	60.3	–	A C
Mmb562	*Mmb. album* and *Methylosarcina*	ATGGTAATGACCCTGGCTGACTTG	24	60.6	New	T
Ia193	Type I a (*M.bacter-M.monas-M.microbium*)	GACTGGAAAGATAGACGTCTATGGG	25	57.8	New	T C G
Ia575	Type I a (*M.bacter-M.monas-M.microbium-M.sarcina*)	TGGCTGACTTGCAAGGTTACCAC	23	61.3	New	A TC A/T T
Nc_oce426	*Nitrosococcus oceani*	CTTGGATGCCATGCTTGCGA	20	59.8	–	
Mth413	*Methylothermus*	CACATGGCGATCTTTTTAGACGTTG	25	58.3	–	
Mc396	*Methylococcus*	CCCTGCCTCGCTGGTGCC	18	64.4	–	C A
501-286	*Methylococcus*-related marine and freshwater sediment clones	GTCAGCCGTGGGGCGCCA	18	66.7	New	C
Mcl408	*Methylocaldum*	GGTTCCGGGTGCGATTTTG	19	57.8	–	A A G A
M90-253	*M.coccus-M.caldum* related marine and freshwater sediment clones	GCTGCTGTACAGGCGTTCCTG	21	61.7	New	
M90-574	*M.coccus-M.caldum* related marine and freshwater sediment clones	ATCGCCGACCTGCTGGGTTA	20	62.2	New	
fw1-641	*M.coccus-M.caldum* related marine and freshwater sediment clones	AGGGCACGCTGCGTACGTT	19	63.3	New	
Ib453	Type I b (*M.thermus-M.coccus-M.caldum* and related)	GGCAGCTACCTGTTCACCGC	20	61.7	–	T G
Ib559	Type I b (*M.thermus-M.coccus-M.caldum* and related)	GGCATGCTGATGTCGATTGCCG	22	60.5	–	C C C
Mcy413	*Methylocystis*	TTCCGGCGATCTGGCTTGACG	21	63.2	New	C C
Mcy522	*Methlocystis* + peat clones	GGCGATTTGCGGCGTTCCA	18	62.3	New	C
Mcy255	*M.cystis parvus*/*echinoides*/strain M	GGCGTCGCAGGCTTTCTGG	19	62.3	New	
Peat264	peat clones	GGCGTTTTTCTGGGTCAACTTCC	23	60.3	–	
Msi520	*Methylosinus trichosporium*	GCGATCGCGGCTCTGCA	17	61.6	–	
Msi269	*Methylosinus trichosporium*	TCTTCTGGGAGAACTTCAAGCTGC	24	60.6	New	C
Msi294	*Methylosinus*	GTTCGGCGCGACCTTCGC	18	62.5	New	T AC TCT
Msi232	*Methylosinus*	ATCCTGGGCGTGACCTTCGC	20	63.3	–	T C G TG
II509	Type II	CGAACAACTGGCCGGCGAT	19	61.7	New	
II630	Type II	CATGGTCGAGCGCGGCAC	18	62.4	New	G CA G A
B2rel251	*Methylocapsa*-related clones	CCGCCGCGGCCCAGTATTA	19	63.4	New	
B2-400	*Methylocapsa*	ACCTCTTTGGTCCCGGCTG	19	60.5	–	
B2all343	*Methylocapsa* and related clones	AACCGCTACACCAATTTCTGGCG	23	61.8	–	A GT C A
pmoAMO3-400	clone pmoA-MO3	ACCCAGATGATCCCGTCGGC	20	62.6	–	G TTG T
RA14-594	RA14 related clones	CCACAACGTTCGTACCTCGA	20	57.9	New	
xb6-539	Novel *pmoA* copy of type II and related environmental clones	AGGCCGCCGAGGTCGAC	17	63.0	–	A T
LP21-190	Novel *pmoA* copy of type II and related environmental clones	ATCGACTTCAAGGATCGCCG	20	58.2	–	
LP21-232	Novel *pmoA* copy of type II and related environmental clones	ATCGTCGCCATGTGCTTCGC	20	61.9	–	
LP21-260	Novel *pmoA* copy of type II and related environmental clones	CGCAGTCCTTCTTCTGGACG	20	58.6	New	G
NmNc533	*Nitrosomonas-Nitrosococcus*	CAACCCATTTGCCAATCGTTGTAG	24	58.6	–	G C
Nsm_eut381	*Nitrosomonas eutropha*	CCACTCAATTTTGTAACCCCAGGTAT	26	59.0	–	
PS5-226	*Nitrosomonas-Nitrosococcus* related clones	ACCCCGATTGTTGGGATGATGTA	23	59.9	–	
Pl6-306	*Nitrosomonas-Nitrosococcus* related clones	GGCACTCTGTATCGTATGCCTGTTAG	26	60.5	–	
NsNv207	*Nitrosospira-Nitrosovibrio*	TCAATGGTGGCCGGTGG	17	58.5	–	T
NsNv363	*Nitrosospira-Nitrosovibrio*	TACTGGTGGTCGCACTACCC	20	59.6	–	C G C A T T
Nit_rel223	AOB related clones	GTCACACCGATCGTAGAGGT	20	56.9	–	
Nit_rel351	AOB related clones	GTTTGCCTGGTACTGGTGGG	20	59.2	–	
Nit_rel470	AOB related clones	CGATATTCGGGGTATGGGCG	20	58.4	–	A
Nit_rel304	AOB related clones	CGCTCTGCATTCTGGCGCT	19	61.8	–	
M84P105-451	environmental clones of uncertain identity	AACAGCCTGACTGTCACCAG	20	58.1	–	
WC306_54-385	environmental clones of uncertain identity	AACGAAGTACTGCCGGCAAC	20	59.2	–	
M84P22-514	environmental clones of uncertain identity	AACTGGGCCTGGCTGGG	17	61.0	–	
gp23-454	environmental clones of uncertain identity	AACGCGCTGCTCACTGCG	18	62.3	–	
MR1-348	environmental clones of uncertain identity	AATCTTCGGTTGGCACGGCT	20	61.1	–	
gp391	environmental clones of uncertain identity	ATCTGGCCGGCGACCATG	18	61.1	–	A TTGCC
gp2-581	environmental clones of uncertain identity	ACATGATCGGCTACGTGTATCCG	23	60.0	–	
RA21-466	clone RA21 – environmental clone of uncertain identity	CGGCGTTCTTGGCGGCAT	18	62.4	–	
mtrof173	Universal	GGbGACTGGGACTTCTGG	18	58.2	–	
mtrof362-I	Methanotrophs	TGGGGCTGGACCTACTTCC	19	59.5	–	
mtrof661	Methanotrophs	GGTAARGACGTTGCKCCGG	19	61.9	–	

a. Numbers at the end of the probe names refer to their relative positions on the pmoA gene of Methylococcus capsulatus (Bath).
b. Sequences are of the sense strand.
c. Oligonucleotide probes developed in the present study are indicated by New.
d. Nucleotide residue(s) at mismatch position(s).

Probe BB51-302 was derived from probe BB51-299 by shifting to the 3′ direction, This way, the GC content of the probe is not concentrated near the (immobilized) 5′ end any longer, resulting in an improved hybridization capacity. Mb_SL#1-418 was derived from Mb_SL#1-421 by extending its 3′ end with 3 nts. This increased its melting temperature and hybridisation capacity to fit into the probe set. Mmb562 was designed *de novo* to ensure reliable detection of *Methylomicrobium album* and *Methylosarcina*, in combination with Mmb303 which detects *Methylomicrobium album* only. Ia193 and Ia575 were derived from Ia193 (old version) and Ia577 respectively. These two probes are targeting the *Methylomonas* – *Methylobacter* – *Methylomicrobium* – *Methylosarcina* subgroup of type I methanotrophs ("type Ia"). Both probes were extended to increase their melting temperatures and hybridisation capacity, thus minimizing false negative calls. 501-286 was originally only 15 nt long with a high GC content and a correspondingly high (59.0°C) melting temperature, theoretically fitting into the probe set. Validation results showed that the hybridisation capacity of this probe was too low. As a compromise it has been extended to 18nt in length with a melting temperature of 66.7°C. Probes M90-253 and M90-574 have been designed *de novo* to target a group of *Methylocaldum*-related environmental clones found in rice rhizosphere (Horz *et al*., 2001). fw1-641 was derived from fw1-639 by shifting in the 3′ direction by 2 nts. This way the position of a discriminative mismatch was shifted from the 3′ end towards the middle of the probe, significantly increasing specificity. Mcy413 was derived from Mcy409 by shifting 4 nts in the 3′ direction, thus avoiding the formation of a stable hairpin structure which interfered with hybridisation in an unpredictable manner. Mcy522 and Mcy255 were designed *de novo* to improve the resolution of the detection of *Methylocystis*. Msi269 was derived from Msi270 (Msi270 had previously been incorrectly named as its position was in fact 267, not 270) by shifting two positions to the 3′ direction and extending by a further nucleotide. This way the formation of a stable hairpin structure was avoided. Msi294 was designed *de novo* to improve the detection of *Methylosinus*. II509 and II630 were derived from II510 and II630, respectively, by extending them by two nucleotides. These probes were only 17 and 16 nt in length which, as discussed above, was too short to provide sufficient hybridisation capacity. RA14-598 was shifted by 4 nts in the 5′ direction, resulting in the new probe RA14-594. This changed the position of two discriminative mismatches (against a large number of type II methanotrophs) from the 5′ end to internal mismatches, significantly increasing specificity. LP21-260 was designed *de novo* to improve the discrimination of a group formed by environmental clones and novel *pmoA*-like genes recently discovered in type II methanotrophs (Yimga *et al*., 2003). Positive results using probes LP21-190, LP21-232 and LP21-260 with *Methylocystis parvus* OBBP and *Methylocapsa acidiphila* B2 indicated the presence of such novel *pmoA*-like genes in these strains. Similar positive hybridisation signals with the remainder of the type II methanotrophs tested are lacking because in those cases, cloned (not novel) *pmoA* products were used as template for target preparation instead of genomic DNA.

The hybridization behaviour of the probe set was predicted based on weighted mismatches, calculated with CalcOligo version 2.03 (http://www.diagnostic-arrays.com/calcoligo/index.htm). The parameters used to weigh mismatches were those found to yield the best predictions. Figure 1 shows the predicted hybridization behaviour of the probe set and the validation results obtained. This new data analysis approach (for hybridization prediction and for determining positive/negative hybridizations) has significantly improved prediction of the behaviour of the probes in hybridization. We would like to emphasize that in our previous publication (Bodrossy *et al*., 2003) positive hybridizations were determined at a signal above 5% of that for probe mtrof173 and mismatch values were indicated only for these and for negative hybridizations with a perfect match or with a single mismatch.

Out of the 68 probes in the probe set, we were unable to obtain reference target DNA for seven which were thus not possible to validate. Most (43) probes displayed hybridization behavior as predicted. The unexpected positive hybridisation results displayed by five probes (Mb292, 501-286, Msi294, Msi232 and gp391) can most likely be explained by the position of the mismatches. For all unexpected false positive results with these probes, there was always a long, uninterrupted perfect match region left, with a calculated T_m of at least 48°C. These perfect match regions appear to be capable of forming stable hybrids under the conditions applied. This finding highlights the need for a nearest neighbor method based hybridization prediction. The new version of CalcOligo (Csontos and Bodrossy, 2003), currently under development, will include this function as well.

Sixty one of the 68 probes were successfully validated. Of the 3660 (61 validated probes x 60 reference strains/clones) individual hybridization reactions, 3621 (98.9%) gave the expected result by either showing signal above threshold limit where expected or by no hybridization where a negative result was predicted. Only 39 of the hybridization reactions (1.1%) resulted in an unpredicted positive or negative hybridization. This success rate of prediction can be further improved by the introduction of a nearest neighbor method based calculation as indicated above.

The evaluation of hybridization results with environmental DNA samples is based on the validation results displayed in Fig. 1, thus considering the real hybridization behaviour of the probe set. This, combined with the rela-

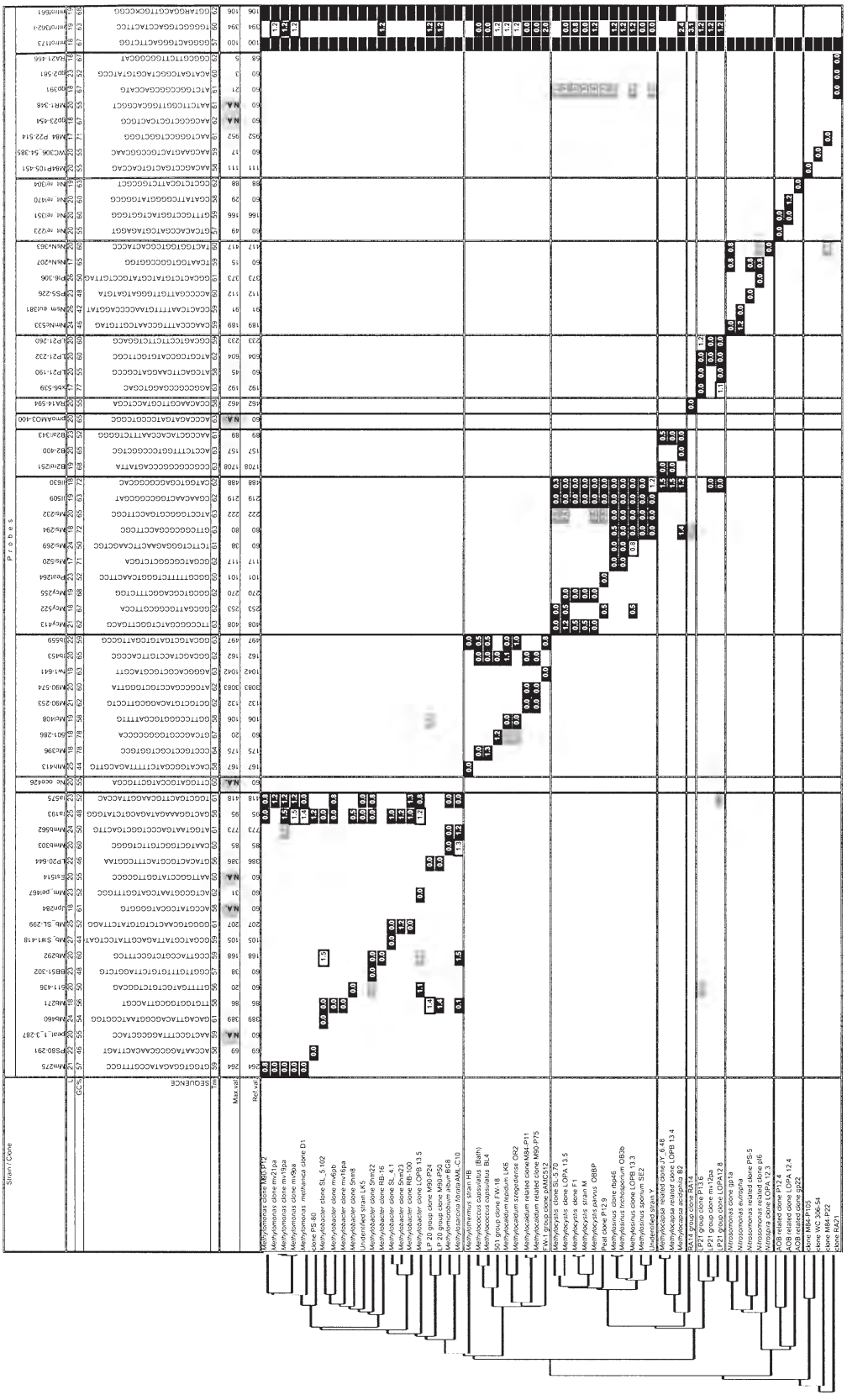


Fig. 1. Range of strain coverage for oligonucleotide probes targeting *pmoA/amoA* genes of methanotrophs, ammonia oxidizing bacteria and bacteria carrying *pmoA/amoA* homologues. A similar table with all 811 sequences considered (without hybridization results) is available from the authors upon request. Under 'Probes' black fill indicates expected positive results, grey fill indicates positive results not predicted and thick black framing indicates negative results where hybridization was predicted. Primers mtrof173 and mtrof 661 contain ambiguous residues and the majority of the *pmoA* database does not cover them, thus mismatch values are not displayed for them. Further, they are in most cases used as PCR primers to generate the target. White numbers inside black and grey boxes indicate the number of weighted mismatches as described in the relevant section of *Results*. Names of environmental clones are preceded by an indication of their predicted immediate phylogenetic relationship. A *pmoA* DNA tree is added to the left providing further insight into the phylogenetic relationships of the reference set. The scale bar indicates an estimated number of 0.1 base changes per nucleotide sequence position.

tively high success rate of hybridization prediction and with the redundant and hierarchical nature of the probe set, guarantees an accurate community analysis. The method offers considerable advances in previous approaches which have used analysis of DNA samples by construction of *pmoA* libraries and sequencing of representative *pmoA* clones (Nold *et al*., 2000; Bourne *et al*., 2001; Pacheco-Oliver *et al*., 2002).

Landfill site simulation experiments

Landfill site simulating lysimeters with different plant covers were monitored for two years and analysed with respect to their methane oxidation potential. The different vegetations covering these simulated landfill site lysimeters investigated were poplar, *Miscanthus*, grass (10% *Lolium perenne*, 20% *Festuca rubra commutata*, 15% *Festuca rubra trichophylla*, 15% *Festuca rubra genuina*, 10% *Festuca ovina*, 15% *Festuca ovina duriuscula*, 15% *Poa pratensis*), a mixture of grass and alfalfa and bare soil as control.

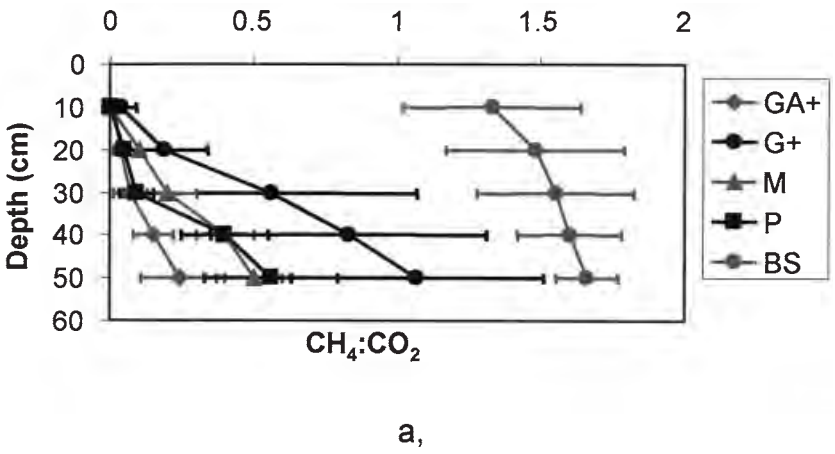
All plant covers tested improved the methane oxidation potential of the soil significantly compared with bare soil. The methane oxidation capacity of the soils is best indicated by the disappearance of methane and the concomitant increase in the concentration of carbon dioxide, with decreasing depth. This is reflected in the methane to carbon dioxide ratios, as shown in Fig. 2. Typical gas concentration profiles for landfill site simulating and control (without biogas added) lysimeters are shown in Fig. 2. Whereas all plant covered lysimeters oxidized most of the methane supplied, thus their maximal methane oxidation capacity was not measured, the methane to carbon dioxide profiles showed strong differences. The highest methane oxidation rate was found in soils with a grass and alfalfa (GA) plant cover. Soils with poplar (P) and with *Miscanthus* (M) oxidized methane at a similar rate. Lysimeters with a grass only (G) plant cover oxidized methane the least, however, methane oxidation was considerably better than observed with bare soil cover (BS).

All biogas-plus lysimeters with plant cover oxidized most of the methane supplied, equaling a methane oxidation rate of 35 g methane m^{-2} day^{-1} or higher. Although the highest methane oxidation capacity of the lysimeters was not tested, these results still correlate well with the extremely high methane oxidation rates reported for landfill site cover soils earlier, 45 g methane m^{-2} day^{-1} (Whalen *et al*., 1990) and 166 g methane m^{-2} day^{-1} (Kightley *et al*., 1995). In contrast, bare soil lysimeters oxidized significantly less methane, at a rate of 25 g methane m^{-2} day^{-1} or less. The difference between the methane oxidation capacity of the planted and bare soil lysimeters is even higher than indicated by these data. Bare soil lysimeters had methane concentrations of 30–35% in the top 10 cm layers, indicating that they reached their maximum oxidation capacity. In contrast, planted lysimeters oxidized all or almost all the methane supplied and thus higher maximum methane oxidation rates can be extrapolated for them.

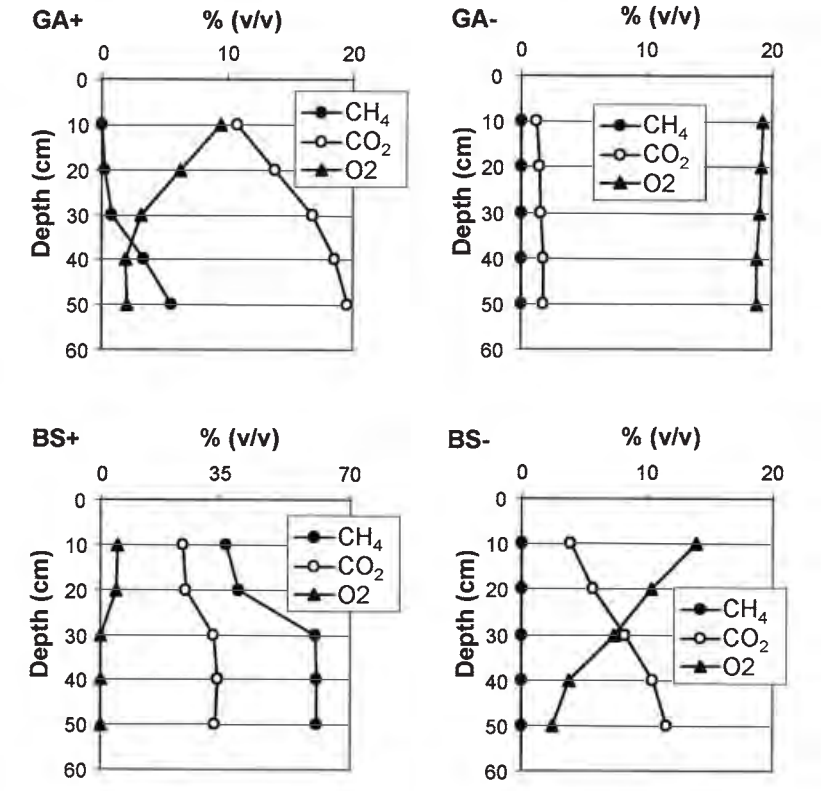
Methanotroph community analysis

Quantification of methanotrophs in relation to the total microbial community was not attempted, however, yields of methanotroph specific PCR reactions under standardized conditions do carry important information on the relative abundance of different types of methanotrophs. In general, DNA extracted from planted lysimeters which received biogas gave high yields with the *pmoA* specific PCR primers whereas those DNA samples from lysimeters without biogas gave very little or no product. The exception were the GA and the BS lysimeters where DNA from the biogas-free control lysimeters also gave significant PCR yields of *pmoA*. In case of the biogas-free controls M and G, only one out of 15 environmental DNA samples gave any *pmoA* PCR product. The top and bottom samples from the planted, biogas fumigated lysimeters gave less *pmoA* PCR products than samples from the middle layers (10–40 cm depths). BS lysimeters (both those with and without biogas treatment) yielded the strongest PCR products from the top layers with a gradual decrease with depth. As the PCR reactions were not competitive and the focus of the method was to generate products wherever a template was present, it is important not to over-interpret these non-quantitative data. Nevertheless it appears that fumigation with methane significantly increased the relative abundance of methanotrophs in planted lysimeters. A similar increase, although expected, was not observed in unplanted lysimeters. Also, a peak of relative methanotroph abundance around 10–40 cm depths in planted, biogas fumigated lysimeters was clearly indicated.

Figure 3 shows the averaged results for all samples which yielded *pmoA* PCR products. Type Ia methanotrophs (*Methylomonas, Methylomicrobium, Methylobacter, Methylosarcina* and related) appeared only in BS samples with biogas (BS +). Besides the general probes for Type Ia (Ia193 and Ia575), only probes BB51-302 and Mb292 were positive in the 20–30 cm depths, probe Mmb562 in the 30 cm and probe Mb271 in the top (10 cm) layer. Considering the specificities of these probes (see Fig. 1), *Methylobacter* sp. BB51-related methanotrophs were detected in 20–30 cm depths, *Methylomicrobium* was just detectable at 30 cm while the 10 cm layers harboured a small population related to another subgroup of *Methylobacter*, previously found in various environmental samples (Baker *et al*., 2001).



a,



b,

Fig. 2. A. Depth profiles of methane to carbon dioxide ratios in the lysimeters. The figure shows results obtained before dismantling the lysimeters. Data shown represent the average of results from five parallel lysimeters. Very similar results were obtained throughout the three summer months preceding dismantling. The ratio of methane to carbon dioxide in the original artificial biogas mix introduced in the bottom of the lysimeters was 3 : 2.
B. Typical gas concentration profiles for landfill site simulating and control lysimeters.
GA + = Biogas-plus lysimeter with Grass-alfalfa mixture cover, GA- = Biogas-free lysimeter with Grass-alfalfa mixture cover, BS + = Biogas-plus lysimeter without plant cover, BS- = Biogas-free lysimeter without plant cover.

Type Ib methanotrophs appeared to be present in almost all samples, including the negative controls. In almost all samples, it was only probe Mcl408 and the general type Ib probes (Ib453, Ib559) which were positive. Mcl408 is specific for the genus *Methylocaldum*, consisting of thermotolerant and thermophilic species(Bodrossy *et al*., 1997). Considering that the biogas free controls yielded very little *pmoA* PCR products, which were then concentrated to yield the observed signal, it is likely that some *Methylocaldum* cells survived in the biogas free lysimeters in a low activity or inactive state. The compost used to fill the lysimeters contained most likely a large population of *Methylocaldum* as indicated by the analysis of samples from a similar composting site (data not shown). In contrast, in the biogas plus lysimeters a high abundance of methanotrophs was present (as indicated

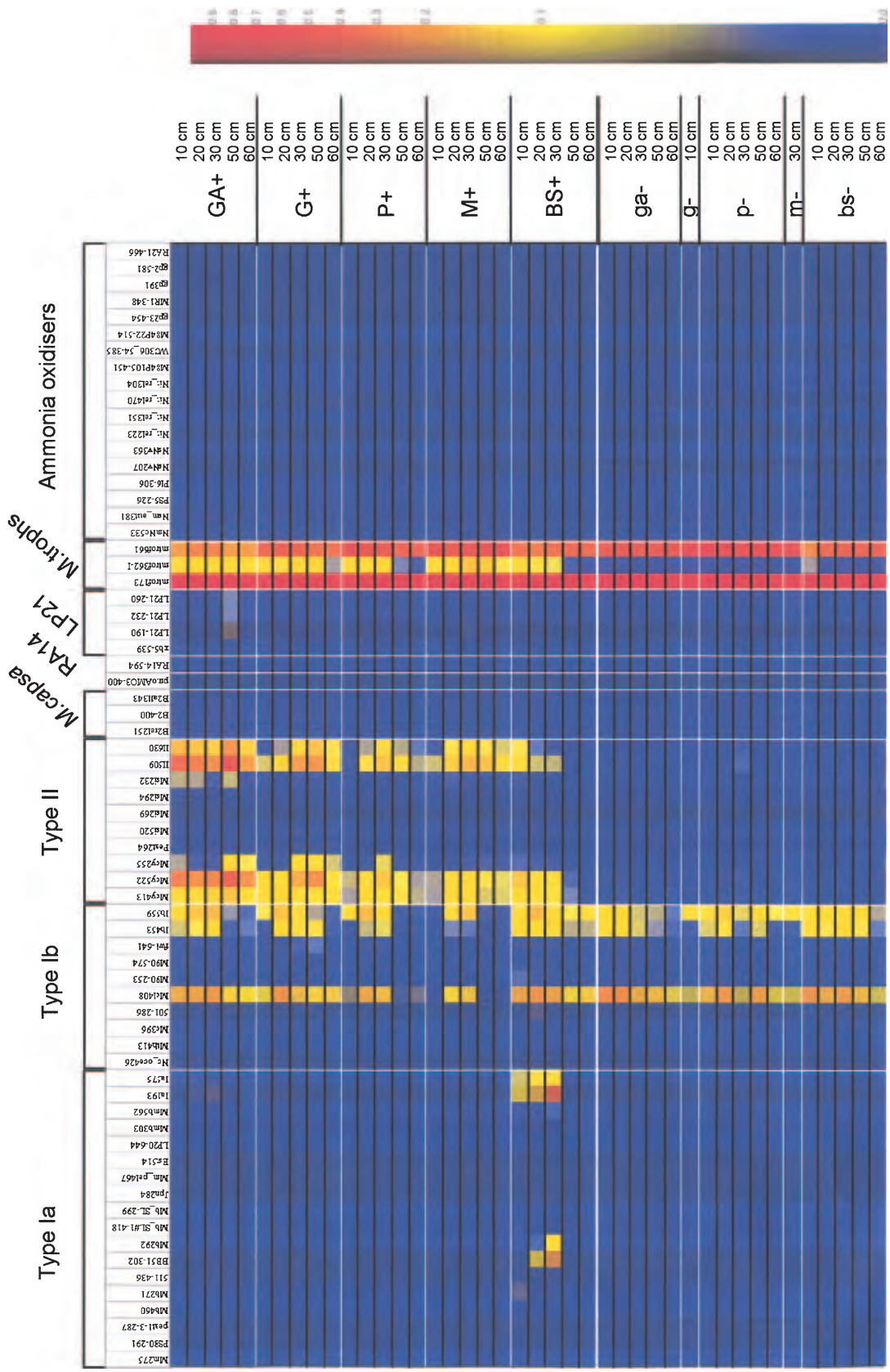


Fig. 3. Methanotroph community analyses. Results of individual microarray experiments were first normalized to positive control probe mtrof173, then to the reference values determined individually for each probe (see *Experimental procedures* for details), averaged between parallels and displayed using the GeneSpring software. In essence, a value of 1.0 indicates maximum achievable signal for an individual probe, whereas 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. GA = Grass-alfalfa mixture, G = Grass, P = Poplar, M = *Miscanthus*, BS = Bulk soil; + and – refer to biogas-plus and biogas-free lysimeters respectively.

by the high PCR yields and by the significantly higher temperatures inside the biogas plus lysimeters). Thus, a strong signal of Mcl408 in these samples indicated an active, major population of *Methylocaldum*. A weak fw1–641 signal in the G + samples from 20 to 30 cm layers showed that a minor methanotroph population was present, related to a group represented by environmental clones from marine and freshwater sediments (Nold *et al*., 2000). Finally, probes 501–286 and M90-253 are specific for two further similar methanotroph groups. Both of these probes were slightly positive in the top layer (20 and 10–20 cm, respectively) of BS + samples indicating the presence of minor populations of these types of methanotrophs.

Type II methanotrophs seemed to dominate all biogas plus lysimeters, but were almost completely absent from the biogas minus control samples. Probes II509 and II630 are general type II probes. Msi232, because of its unexpected positive hybridization with almost all *Methylocystis* strains tested was also considered to be a general probe for type II methanotrophs. All the type II diversity in the lysimeter samples was contributed by members of the *Methylocystis* genus (probes Mcy413, Mcy522 and Mcy255). Interestingly, no *Methylosinus* specific signal was obtained. The depth profile of *Methylocystis* differed between different plant vegetations. GA + lysimeters harboured a major *Methylocystis* population throughout the entire depth. The top (10 cm) layer of the G +, P + and M + lysimeters and the bottom (60 cm) of the P + lysimeter contained only very little *Methylocystis*. BS + lysimeters had a clear gradient with the highest abundance of *Methylocystis* in the top layer, gradually disappearing by the 50 cm layer. *Methylocystis* can be divided into two subgroups, the first one being specifically targeted by Mcy522, and the second one by Mcy255. Mcy413 targets both groups. The significance of these two groups is unclear, but they can be easily differentiated using these probes and form coherent branches on *pmoA* trees (data not shown). The first group is a diffuse one with no representatives identified at the species level. The second group consists of relatives of '*Methylocystis echinoides*', *Methylocystis parvus* and *Methylocystis* strain M. The first group of *Methylocystis* was the dominant one, contributing the majority of *Methylocystis* signal in all samples. The second group of *Methylocystis* appeared to be more selective, present only in the top (10 cm) and bottom (50–60 cm) layers of GA +, the middle and bottom (30–60 cm) layers of G + and the middle layer (30 cm) of P + 0. M + and BS + samples seemed to harbour very little or no population of this *Methylocystis* group. The environmental significance of this finding is at present unclear and it is hoped that the accumulation of similar findings will eventually indicate the environmental niches filled by different subgroups of methanotrophs.

The LP21 probes (LP21-190, LP21-232 and LP21-260) were designed against a group which, until very recently, was considered to represent a lineage of uncultivated bacteria with uncertain physiological function. Recently it was shown (Yimga *et al*., 2003) that type II methanotrophs harboured a second, unusual copy of *pmoA* genes, comprising this group or at the least, belonging to it. Weak signals were obtained from the middle layers (30–50 cm) of planted, biogas plus lysimeters. These signals were not reproducible however, i.e. one or two of the three lysimeters analysed for GA +, G +, P + and M + gave these positive signals whereas the other parallel samples did not (this is why it is not seen on Fig. 3 where average results from the parallel lysimeters are shown). Signals correlated in most cases with high abundance of the second *Methylocystis* subgroup (consisting of relatives of '*Methylocystis echinoides*', *Methylocystis parvus* and *Methylocystis* strain M) as indicated by strong signals for probe Mcy255.

The dominant methanotroph populations in all biogas plus lysimeters were type II *Methylocystis* and type Ib *Methylocaldum*. *Methylocaldum* related methanotrophs were also present in biogas free lysimeters, indicating their ability to survive under methane free/low methane conditions (there was probably some indigenous methane production in the deep layers of the biogas-free lysimeters). GA + lysimeters, oxidizing methane the most efficiently, harboured the highest abundance of *Methylocystis* throughout the depth profile of the lysimeters. The top (10 cm) layers of P + and M + lysimeters displayed the lowest diversity and abundance of methanotrophs. The contrasting high diversity in the same layers of GA + and G + lysimeters is probably attributable to the very high density grass root system ('root mat') at this depth, potentially transporting oxygen to the bacteria. The similarly low diversity and abundance of methanotrophs in the same lysimeters in the bottom layers (50–60 cm) is, however, unexpected and hard to explain. BS + lysimeters showed a high diversity in the 10–30 cm layers. This was comprised of type Ia *Methylobacter* sp. BB51-related and *Methylomicrobium* strains, type Ib *Methylocaldum* and type II *Methylocystis*. At the deeper (50–60 cm) layers, only Type Ib *Methylocaldum* related methanotrophs were detected.

Results from individual lysimeter samples (Fig. 4) showed significant variation for probes targeting methanotroph groups found at low frequency (type Ia, type Ib except *Methylocaldum*, LP21 probes). Some of these indicate a variation between the parallel lysimeters, where the variation is consistent through more sampling depths. For example, the GA- lysimeter #1 gave positive signal for probes 501–286, Mcy413 and Mcy522 at depths of 20, 50 and 60 cm. These indicate the presence of an environmental type Ib group of methanotrophs found in sediments

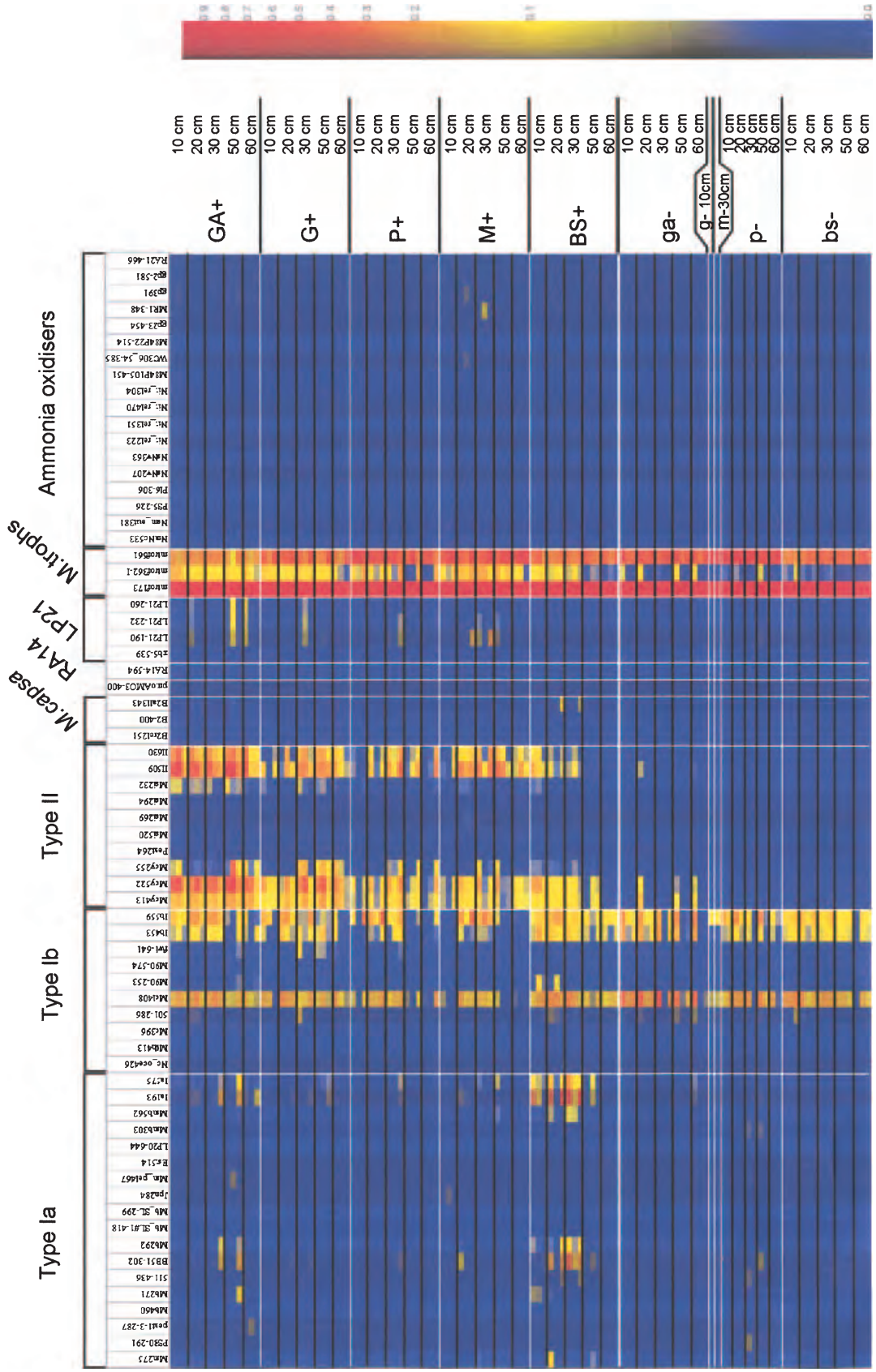


Fig. 4. Methanotroph community analyses. Individual results are shown. Results were first normalized to positive control probe mtrof173, then to the reference values determined individually for each probe (see *Experimental procedures* for details) and displayed using the GeneSpring software. In essence, 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. Colour coding is indicated on the side bar. GA = Grass-alfalfa mixture, G = Grass, P = Poplar, M = *Miscanthus*, BS = Bulk soil; + and – refer to biogas-plus and biogas-free lysimeters respectively.

(Nold *et al*., 2000) and that of *Methylocystis*, throughout the lysimeter. Similarly, results from GA + #3 show a significant *Methylobacter* population at 30 and 50 cm; BS + #3 has a positive signal for probe B2all343 (targeting *Methylocapsa acidiphila* and related *pmoA*) at 10 (very weak), 20 and 30 cm; BS + #2 is positive with probe M90-253 at 10 and 20 cm. Other variations appear only in individual samples. Whereas a false positive result can never be excluded, most of these probably represent true variations and indicate the importance of parallel assays if reproducible trends concerning soil microbial communities are to be detected. Results for probes targeting methanotrophs dominant in the samples were, on the other hand, rather consistent. The dominance of *Methylocaldum* and *Methylocystis* (the latter only in biogas plus samples) and the significant appearance of type Ia methanotrophs (*Methylobacter*) only in BS + can be clearly concluded from the individual results as well.

The potential of the microarray technology to diagnose environmental changes was demonstrated by an accidental result. A GA + lysimeter (GA + 20) displayed a most unusual pattern of methanotroph diversity (Fig. 5). Checking the gas profile revealed that there had been a major fault in the lysimeter, most likely a gas leakage near to the bottom (70 cm), where biogas was added. Besides the diversity found in normal GA + lysimeters, significant diversity of type Ia and type Ib methanotrophs was detected. This covered the groups *Methylomonas* (Mm275), *Methylobacter* sp. BB5.1 (BB51-302), *Methylomicrobium* (Mmb562), *Methylococcus* (Mc396) and three groups represented by environmental clones from marine and freshwater sediments (Nold *et al*., 2000) and rice fields (Horz *et al*., 2001), represented by probes 501–286, M90-253 and fw1–641.

The competition between type Ia and type II methanotrophs followed the trends described previously (Graham *et al*., 1993; Amaral and Knowles, 1995; Henckel *et al*., 2000b; Macalady *et al*., 2002). Although the conditions in general favoured type II methanotrophs, type Ia methanotroph populations competed more efficiently with type II methanotrophs under relatively high oxygen and low methane partial pressures. These conditions were fulfilled by the top layers of the BS + lysimeters and by the bottom layers of GA + 20. In the former case there was no air conducted down to the bottom layers by plant roots, thus type II methanotrophs could not consume the majority of the methane by the time it reached the top layers. In the latter case, the gas concentration ratios were shifted towards low methane – high oxygen, most likely by a gas leakage. Nitrogen limitation (N_2 fixation by type II methanotrophs) was unlikely to play a role in this competition since both NO_3^- and NH_4^+ concentrations were in the range of 0.2–20 mg l^{-1} at the end of the experiment which would repress N_2 fixation by methanotrophs.

Conclusions

Probes of the microarray were successfully improved by one of the following ways. Probe positions were shifted if discriminative mismatches or the majority of the GC residues were near the 5′ or 3′ end. The formation of stable hairpin structures was also avoided by shifting the position of the probes. Short (15 and 16 nt long) probes were extended to 18 nt, even at the expense of increasing their T_m above that of the probe set. In one case even a 17 nt long probe had to be extended.

A high methane oxidation capacity of 35 g methane m^{-2} day^{-1} or higher was found in all biogas plus lysimeters with plant cover. The best performance, as indicated by the gas profiles, was found in lysimeters with a grass and alfalfa mixture (GA +) which correlated with a high ratio of *Methylocystis* throughout the soil layers analysed. Poplar (P +) and *Miscanthus* (M +) plant covers also supported methane oxidation effectively while grass alone (G +) exerted a significantly less positive effect. All four vegetations did, however, show a drastic positive effect on methane oxidation compared with bare soil. Our results indicated a link between community structure and methane oxidation capacity, at least when planted and unplanted lysimeters were compared. The more subtle differences exerted by the different plant covers on methane oxidation and methanotroph community structure are not clearly linked. This is especially true if GA + is compared with G + where the methane oxidation potential differed the most whereas the community structures were the most similar. Clearly, further studies need to address the activity of the individual layers by methane removal measurements and/or *pmoA* mRNA based assays.

Besides *Methylocystis*, *Methylocaldum* was found to be a dominant member of the methanotroph community in virtually all of the lysimeter samples. This was true for the biogas-free controls as well, even though the absolute amount of methanotrophs was much lower in these samples. Earlier work on landfill site methanotrophs did not find *Methylocaldum* which is probably caused by the narrow specificity of the PCR primers used in those studies (MethT1bR, MethT1cR and MethT1dF) (Wise *et al*., 1999) and to the lack of PLFA analysis data on this group of methanotrophs.

Type Ia methanotrophs (*Methylomonas, Methylomicrobium, Methylobacter, Methylosarcina* and related methanotrophs) were found only in BS + samples and a GA + lysimeter with an air leakage. This is in line with earlier findings that type Ia methanotrophs have a competitive advantage over type II strains under high oxygen and relatively low methane concentrations. Further work is needed to investigate whether the differences detected are caused by the supply of methane and oxygen or other factors.

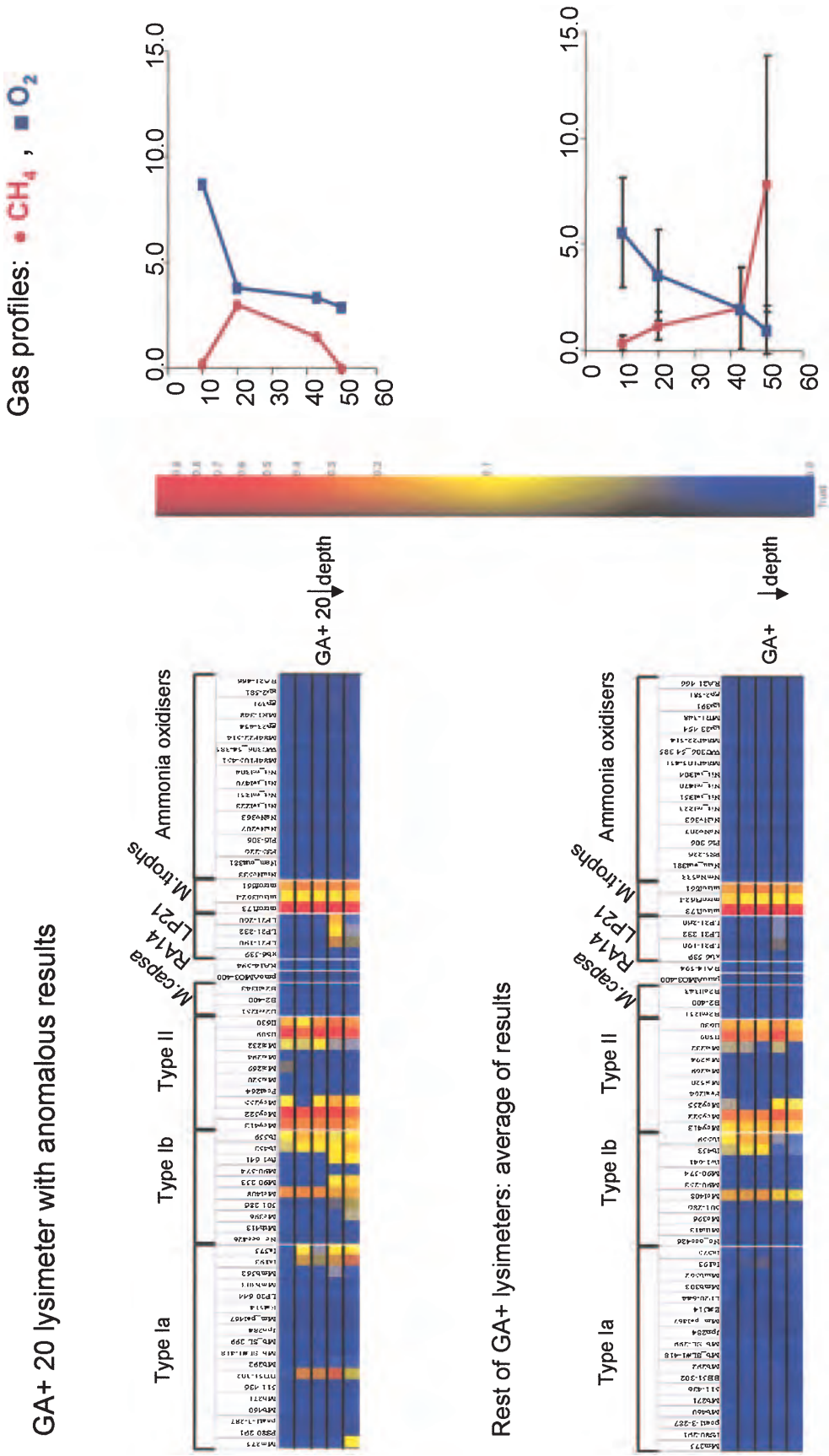


Fig. 5. Methanotroph community profile of lysimeter GA + 20 compared with the average profile of three other GA + lysimeters (data from Fig. 3). Gas depth profiles are also shown. Results of individual microarray experiments were first normalized to positive control probe mtrof173, then to the reference values determined individually for each probe (see *Experimental procedures* for details), averaged between parallels and displayed using the GeneSpring software. In essence, a value of 1.0 indicates maximum achievable signal for an individual probe, whereas a value of 0.1 indicates that about 10% of the total PCR product hybridized to that probe. Colour coding is indicated on the side bar.

Besides the above general trends, considerable variations were found among parallel samples. These indicated differences between the environments developed in the lysimeters as well as local, small-scale heterogeneities. Caution has to be taken when interpreting these results as different geographic locations and different soil characteristics may result in drastic differences in methanotroph communities, as also shown for rice fields (Hoffmann *et al*., 2002). A wider study analysing a range of landfill sites is needed to gain a deeper understanding of the role different methanotrophs play in oxidizing the vast majority of the methane produced there.

Using microbial diagnostic microarrays, it was possible to analyse rapidly bacterial community structures at a high resolution. The resolution of the technique is limited only by the degree of conservation of the gene the probes are designed for and by the probes themselves. It is now possible for one researcher to routinely analyse 40 samples or more per week, from environmental DNA preparation to the analysis of results. With the creation and sequencing of clone libraries the same task would take months. Microarrays cannot, however, completely replace this approach as the probe set is limited to microbes with already sequenced genes. The application of nested probes offers the potential of indicating the presence of novel microbes by a signal at the higher taxonomic level, not matched by corresponding signals from narrower specificity probes. It has to be noted, that the quantification potential of the approach is only suitable for the comparison of similar environments on a semiquantitative basis, because of inherent biases in the PCR and to the differences in *pmoA*/*amoA* copy numbers in different methanotrophs, ammonia oxidizers and functionally related bacteria. The importance of assaying parallel samples and analysing both average and individual results is highlighted by the results of this work. The potential of microbial diagnostic microarrays for the detection of environmental changes and perturbations was demonstrated by the detection of anomalous gas profiles (most likely caused by an erroneous gas leakage) by the analysis of microbial community structures.

Experimental procedures

Lysimeter experiments

Small lysimeters with a volume of 200 l, and a depth of 65 cm were set up to simulate landfill site environments with different plant covers. Lysimeters contained a 15 cm drainage layer of gravel in the bottom, followed by a thin layer of fleece to stop small particles from being washed out into the drainage layer. Finally, lysimeters were filled up with compost made of sewage sludge. Half of the lysimeters received artificial biogas (CH_4:CO_2 = 3 : 2) from the bottom at a constant rate of 100 l day^{-1} m^{-2}. For each plant cover, five lysimeters with biogas supply and four biogas-free control lysimeters were set up. The following plant covers were tested: a mixture of grass (10% *Lolium perenne*, 20% *Festuca rubra commutata*, 15% *Festuca rubra trichophylla*, 15% *Festuca rubra genuina*, 10% *Festuca ovina*, 15% *Festuca ovina duriuscula*, 15% *Poa pratensis*) and alfalfa (GA + and GA -); grass only (G +and G -); *Miscanthus* (M + and M -); poplar (P +and P -) and as negative control bare soil (BS + and BS -), where + and – refer to lysimeters with biogas supply and biogas free controls respectively. Lysimeters were covered on the side in a thick layer of insulation material used in the building industry. Lysimeters contained gas drainage lines at 10, 20, 30, 40 and 50 cm depths for gas sampling. Gas concentrations (CH_4, O_2 and CO_2) were measured by gas chromatography. The compost used to fill the lysimeters had a pH of 7.0, 38% water content, 12.22% total C, 0.82% total N and a C/N ratio of 14.91. Methane concentrations at 10 cm depth in planted, biogas plus lysimeters were reduced from 60% to a value of 0–3%. Bare soil lysimeters oxidized about half of the methane provided, decreasing its concentration from 60% to 30–35%.

Lysimeters were kept running for 18 months before dismantling. Dismantling and sampling occurred in the second week of September 2000 when daily average temperatures were between 14.1 and 19.8°C. A white layer formed in all lysimeters supplied with biogas between a depth of 12–38 cm. Average positions of the white layers for the different plant covers were: GA + –28 cm, G + – 32 cm, M + –30 cm, P + –25 cm, BS + – 15 cm. Soil samples were collected from five depths from each lysimeter (10 cm, 20 cm, the middle of the white layer, 50 cm and 60 cm for GA +, G +, M + and P + and 10, 20, 30, 50, 60 cm for BS + and biogas free controls). About 200–300 g soil samples were collected, mixed and sieved (≤1 mm). Roughly 100 g of the homogenized and sieved samples were lyophilized and stored at 4°C until use.

Oligonucleotide probe design

Database and phylogenetic trees were constructed and oligonucleotide probes were designed using the phylogenetic software package ARB (Strunk *et al*., 2000). A comprehensive database containing all published *pmoA*/*amoA* and related sequences, as well as many unpublished ones was established. Alignments were made using Old Aligner function in ARB_EDIT. Neighbour joining DNA and protein trees were constructed and used to guide the probe design process. Probes were designed using the Probe Design and Probe Match functions, accessing a PT-server database created from the above ARB database. Outputs of the Probe Match function were imported into CalcOligo 2.03 (http://www.diagnostic-arrays.com/calcoligo/index.htm). CalcOligo was used to create an Excel table indicating predicted melting temperatures (based on the nearest neighbour model and SantaLucia parameters), length and GC content of the probes and the number of weighted mismatches between each probe-target pair. Nearest neighbour T_m values were calculated with concentration settings of 250 nmol for oligonucleotide and 50 mmol for Na^+. Factors for weighing mismatches in CalcOligo were as follows. Positions: 5′ 1st 0.3; 5′ 2nd 0.6; 5′ 3rd 1.0; 3′ 1st 0.3; 3′ 2nd 0.8; 3′ 3rd 1.1; all other positions 1.2.

Basepairs: dArC 1.2; dTrC 1.2; dGrU 0.7; dTrG 0.4; all other mismatched basepairs 1.0. Probe-target pairs with weighted mismatch values of up to 1.5 were expected to yield positive hybridization under the conditions applied.

Microarray preparation

Oligonucleotides for immobilization were custom synthesized (VBC Genomics, Vienna, Austria) with a 5′-NH_2 group, followed by a C12 spacer and five thymidines residues preceding the probe sequence. A 384 well flat bottom plate was prepared with 30 μl of 50 μM oligonucleotide solutions in 50% (v/v) DMSO. Samples were spotted with an OmniGrid spotter (1 TeleChem SMP3 pin) at 50% relative humidity (using the humidity controller of the spotter) and 22°C, onto silylated slides (with aldehyde chemistry, Cel Associates, Houston). Arrays were always spotted in triplicate to enable a statistical correction for errors. Spotted slides were incubated overnight at room temperature (22°C) at <30% relative humidity, rinsed twice in 0.2% (w/v) SDS for 2 min at room temperature with vigorous agitation to remove the unbound DNA. Slides were then rinsed twice in distilled water (dH_2O) for 2 min at room temperature with vigorous agitation, transferred into dH_2O, preheated to 95–100°C for 2 min, and allowed to cool at room temperature (~5 min). Slides were treated in a freshly (immediately before use) prepared sodium borohydride solution for 5 min at room temperature to reduce free aldehydes. Preparation of the sodium borohydride solution: 0.5 g $NaBH_4$ was dissolved in 150 ml phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, in 1000 ml H_2O, pH 7.4, autoclaved) then 44 ml of 100% ethanol was added to reduce bubbling. Slides were rinsed three times in 0.2% (w/v) SDS and once in dH_2O for 1 min each at room temperature. Finally, slides were dried individually using an airgun fitted with a cotton wool filter inside (to keep oil microdroplets away from the slide surface). Dried slides were stored at room temperature in the dark before use.

Environmental DNA preparation

DNA was prepared from soil samples using a modification of a method by Yeates and Gillings (1998), based on the FastDNA spin kit for soil (QBiogene). Soil (0.3 g) and 780 μl lysis buffer [100 mM $NaPO_4$ pH 7.0; 1% CTAB; 1.5 M NaCl; 5 mg ml^{-1} lysozyme (added right before use)] was added into a Multimix FastPrep tube and incubated at 37°C for 30 min MT buffer (122 μl) was added and tubes were shaken in the FastPrep instrument for 30 s at 5.5 m s^{-1}. Samples were centrifuged for 15 min at 12 000 *g* and 700 μl supernatant were collected. Lysis buffer (500 μl) and 50 μl MT buffer were added to the FastPrep tubes, extraction was repeated and a second 700 μl of supernatant was transferred into separate Eppendorf tubes. At this step, 2 × 700 μl supernatant was obtained from each sample. Five μl of 10 mg ml^{-1} freshly made proteinase K was added to each tube. Tubes were incubated at 65°C for 30 min Samples were extracted with phenol-chloroform-isoamyl alcohol (25 : 24 : 1), followed by a chloroform-isoamyl alcohol (24 : 1) extraction. One hundred and twenty-five μl of 7.5 M potassium acetate was added, samples were incubated on ice for 5 min and then centrifuged at 12 000 *g* for 10 min. Supernatants (2 × 600 μl per soil sample) were transferred to new tubes, 700 μl Binding Matrix was added and tubes were mixed for 5 min in an Eppendorf shaker. Binding Matrix with bound DNA was pelleted by 1 min centrifugation at 12 000 *g*, supernatant was discarded and pellet was resuspended in 500 μl wash buffer. The resulting suspension was added into a Spinfilter, and centrifuged for 1 min at 10000 r.p.m. Eluate was discarded and pellet was washed again in 500 μl wash buffer. After discarding the second eluate, the Spinfilter was centrifuged for another 10 s to dry the pellet. The filter was taken into a new tube, 200 μl of TE pH 8.0 was added, the filter was incubated at room temperature for 1 min and centrifuged for 1 min at 12 000 *g*. The eluate collected in the catch tube contained the purified DNA which was stored at −20°C until use.

Target preparation

pmoA/*amoA* genes were amplified using the forward primer *pmoA*189 (5′-GGBGACTGGGACTTCTGG-3′) and either one of the reverse primers T7-mb661 (5′-TAATACGACTCACT ATAGCCGGMGCAACGTCYTTACC-3′) or T7-A682 (5′-TAATACGACTCACTATAGGAASGCNGAGAAGAASGC-3′) where B = (CGT), M = (AC), Y = (CT), S = (CG) and N = (ACGT). Primers T7-mb661 and T7-A682 were specific for methanotrophs and for methanotrophs/AOBs/homologous genes from environmental libraries respectively (Bourne *et al*., 2001). The reverse primers contained the T7 promoter site (5′-TAATACGACTCACTATAG-3′) at their 5′ end, which enabled T7 RNA polymerase mediated *in vitro* transcription using the PCR products as templates. For each target, three PCRs of 50 μl volume each, consisting of 1 × PCR buffer, 1.5 mM $MgCl_2$, 50 nM for each four dNTPs, 15 pmol of both primers, 1 ng genomic/environmental DNA or 0.1 ng cloned PCR product as template, and 1 U of Taq polymerase (Invitrogen), were performed in a Hybaid Combi Thermal Reactor TR2 using *Taq* DNA polymerase in accordance with the manufacturer's instructions. Amplification conditions were: 95°C for 5 min before template was added; then 32 cycles of: 1 min at 95°C; 1 min at 58°C for mb661 or at 56°C for *pmoA*682; 1 min at 72°C; followed by a final elongation step of 10 min at 72°C. Polymerase chain reaction products were pooled and purified using the HighPure PCR purification kit (Roche Diagnostics GmbH, Mannheim, Germany), according to manufacturer's instructions. Purified DNA was dissolved in ultrapure water to a DNA concentration of 50 ng/μl and stored at −20°C.

A new, two-step PCR (K. Rudi, pers. comm.) was applied to amplify the *pmoA* and related genes from environmental DNA samples. In the first step, 15 cycles were carried out with composite primers composed of *pmoA*-specific-3′ regions and non-related 5′ head regions (T3c-*pmoA*189 – CAGAGATGCAAAT TAACCCTCACTAAAGGGNGACTGG GACTTCTGG, and T7c-mb661 – CCAAGCCTTCTAATAC GACTCACTATAGCCGGMGCAACGTCYTTACC). The second step consisted of an extra 25 cycles carried out with primers consisting of only the head regions (T3c – CAGAGATGCAAATTAACCCTCACTAAAG and T7c – CCAAGCCTTCTAATACGACTCACTATAG). Exact PCR conditions were as above with the following modifications. As primers, only 1.5 pmoles each of T3c-*pmoA*189 and T7c-

mb661 were added per 50 μl reaction initially. After the completion of the first 15 cycles, 15 pmoles each of primers T3c and T7c were added with an extra 1 U of *Taq* DNA polymerase and a further 25 cycles were carried out. Annealing temperature was 58°C throughout the entire PCR protocol.

This two-step PCR was found to yield community composition results comparable to those obtained with traditional PCR using the *pmoA* specific primers only. Further, it was possible to amplify *pmoA* and related genes from samples which did not give rise to PCR products with the traditional method (data not shown). The applied primer pair was specific for *pmoA* and closely related genes, while they did not amplify *amoA* and related genes. Thus, no signal was expected for ammonia oxidizers, the *Methylocapsa acidiphila* group, the RA14 group, from tundra methanotrophs with a highly divergent *pmoA* gene (Pacheco-Oliver *et al*., 2002) and from RA21 and related sequences (Holmes *et al*., 1999).

Working under RNAse-free conditions, *in vitro* transcription was carried out as follows: 8 μl purified PCR product (50 ng μl^{-1}), 4 μl 5× T7 RNA polymerase buffer, 2 μl DTT (100 mM), 0.5 μl RNAsin (40 U μl^{-1}) (Promega), 1 μl each of ATP, CTP, GTP (10 mM), 0.5 μl UTP (10 mM), 1 μl T7 RNA polymerase (40 U μl^{-1}) (Gibco BRL) and 1 μl Cy3 or Cy5-UTP (5 mM) were added into a 1.5 ml microcentrifuge tube and incubated at 37°C for 4 h. RNA was purified immediately using the Quiagen RNeasy kit according to manufacturer's instructions. Purified RNA was eluted into 50 μl dH_2O. RNA yields and dye incorporation rates were measured by spectrophotometry.

Purified RNA was fragmented by incubating with 10 mM $ZnCl_2$ and 20 mM Tris.Cl (pH 7.4) at 60°C for 30 min. Fragmentation was stopped by the addition of 10 mM EDTA pH 8.0 to the reaction and putting it on ice. RNAsin (1 μl 40 U μl^{-1}) was added to the fragmented target. Fragmented, labelled RNA targets were stored at −20°C. Length of the fragmented RNA target was measured by running the sample on an ABI capillary sequencer as well as running on a thin, 2% agarose gel applied onto a standard microscope slide and subsequent scanning in a GenePix 4000 A scanner.

Reference targets and artificial target mixtures for testing the quantification potential were synthesized by mixing known amounts of purified PCR products and carrying out *in vitro* transcription and target fragmentation as described above.

Hybridization

No prehybridization was done. Hybridization was carried out in a custom tailored aluminum block used as an insert for a temperature controlled Belly Dancer (Stovall Life Sciences, Greensboro, NC, USA) set at maximum bending (about 10°). The hybridization block was preheated to 55°C for at least 30 min to allow the temperature to stabilize. An Eppendorf incubator was also preheated to 65°C. HybriWell (Grace BioLabs) stick-on hybridization chambers (200 μl in volume) were applied onto the slides containing the arrays. Assembled slides were preheated on top of the hybridization block. For each hybridization, 124 μl DEPC-treated water, 2 μl 10% SDS, 4 μl of 50 × Denhardt's reagent (Sigma), 60 μl 20 × SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) and 10 μl target RNA (corresponding to about 400 ng RNA) were added into a 1.5 ml Eppendorf tube and incubated at 65°C for 1–15 min. Preheated hybridization mixtures were applied onto assembled slides via the port in the lower positions (to minimize risk of air bubbles being trapped within the chamber). Chambers were sealed with seal spots (Grace BioLabs) and incubated overnight at 55°C at 30–40 r.p.m. circulation and maximum bending.

Following hybridization, HybriWell chambers were removed individually and slides were immersed immediately into 2 ×SSC, 0.1% (w/v) SDS at room temperature (22°C). Slides were washed by shaking at room temperature for 5 min in 2 ×SSC, 0.1% (w/v) SDS; twice for 5 min in 0.2 × SSC and finally for 5 min in 0.1 × SSC. Slides were dried individually using an airgun with a cotton wool filter inside. Slides were stored at room temperature in the dark and scanned the same day.

Scanning and data analysis

Hybridized slides were scanned at three lines to average, at 10 μm resolution with a GenePix 4000 A laser scanner (Axon, Foster City, CA, USA) at wavelengths of 532 nm and 635 nm for Cy3 and Cy5 respectively. Fluorescent images were captured as multilayer tiff images and analysed with the GenePix Pro 3.0 software (Axon). Microsoft Excel was used for statistical analysis and presentation of results.

Results were normalized to a positive control. Hybridization signal for each probe was expressed as percentage of the signal (median of signal minus background) of the positive control probe mtrof173 on the same array. As each slide contained triplicate arrays, normalized signal intensities of the triplicate spots on a slide were used to determine average results and standard deviations. Several probes produced non-specific background signal up to 3% of their maximum signal (obtained with perfect match targets). Hybridization between a probe and a target was thus considered positive if the signal was at least 5% of the strongest signal obtained for that probe with the validation set of reference strains/clones. For probes, where no perfect match reference target was available or the strongest signal was less, than 60 (% of the signal obtained for mtrof173), this reference value was arbitrarily set to 60. This was found to remove to minimize false positive calls while not creating any false negative call.

Even though no dedicated negative controls were applied, for each individual hybridization over 70% of all probes present on the array were negative controls.

Nucleotide sequence accession numbers

The partial *pmoA* sequences used in this study to validate the probe set are available under accession nos. AB484595, AB484597, AB484601, AB480948, AF148521, AF148522, AF150764, AF177325, AF211872, AF211879, AF211883, AF211889, AF264115, AF239884, AF264136, AF358040, AF358041, AF358045, AF358050, AF358053, AF358054, AF358055, AF368358, AF368373, AJ278727, AJ299947, AJ299948, AJ299951, AJ299955, AJ299957, AJ299963, AJ299964, AJ459006, AY080942, AY080950, AY080955, AY236074, AY236075, AY236076, AY236077, AY236078, AY236079, AY236080, AY236081, AY236082, AY236083, AY236084, AY236085, AY236086, AY236087, AY236517,

AY236518, U31650, U31654, U72670, U81596, U89302, U89303, U89304 and U94337.

Acknowledgements

Work at ARC was supported by the Fonds zur Förderung der wissenschaftlichen Forschung, Austria (Project number P15044), and funding through the EU 6th framework Quality of Life and Management of Living Resources grant QLK-3 CT-2000–01528. Funding from the EU towards COST Action 853 (Agricultural Biomarkers for Array Technology) contributed to the presented results by enhancing exchange of ideas.

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