

Development, validation and application of a diagnostic microarray for methanotrophs

Levente Bodrossy

Microbial diagnostic microarrays at Seibersdorf research

- **Techniques**
- **Pilot array - construction and validation**
- **Pilot array - first high-throughput application**
- **Options to adjust baseline technique**

Microbial diagnostic microarrays at Seibersdorf research

- **Techniques - Microarrays for Beginners, Kluwer, 2003**
- **Pilot array - construction and validation**
- **Pilot array - first high-throughput application**
- **Options to adjust baseline technique**

Aim:

Fast, parallel detection and identification of microorganisms

- Food contamination
 - Human / animal / plant pathogens
- Gut, skin, oral microbial communities (human)
 - Epidemiology studies
- Environmental analyses, ecotoxicology
 - Assessment of soil quality
- Quality control of wastewater treatment
- Analysis of processes by complex microbial communities

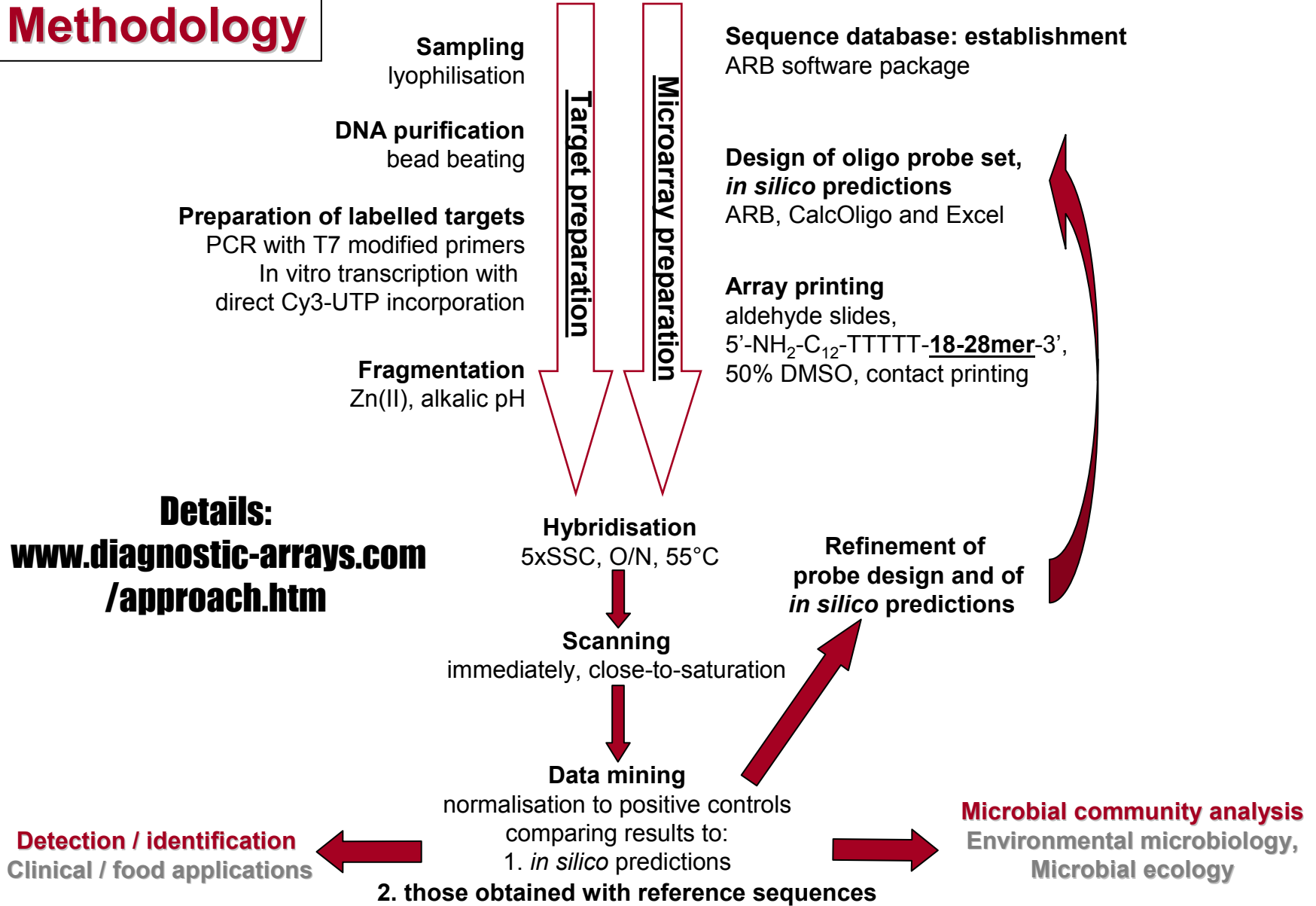
Methodology:

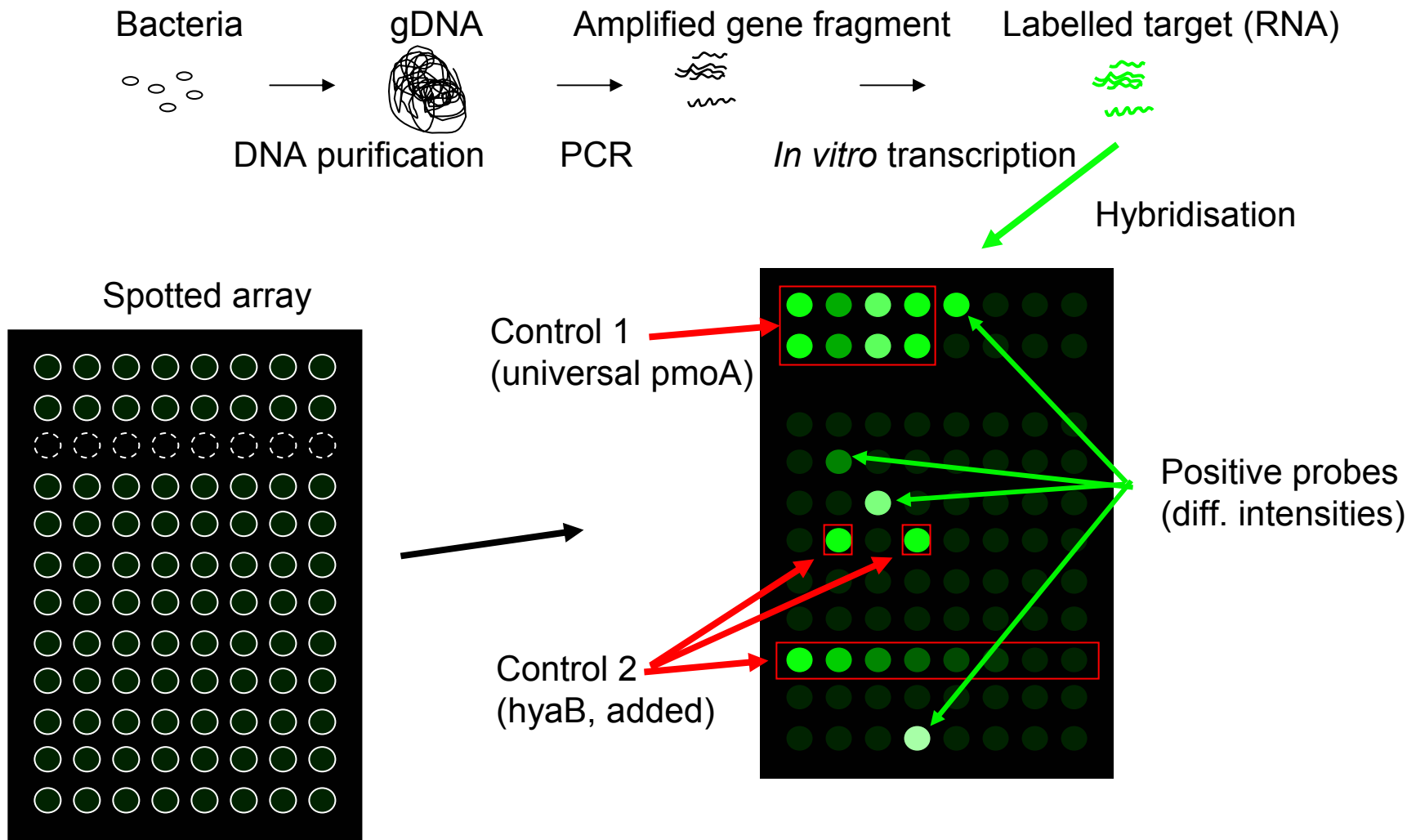
Oligonucleotide microarrays

+

Molecular ecology

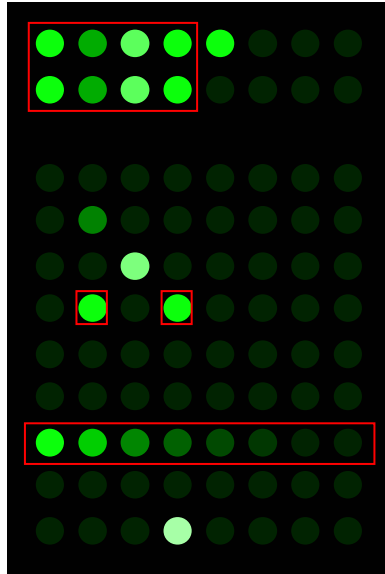
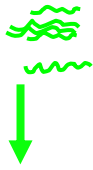
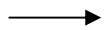
Methodology





Hybridisation with a single strain #1

Bacteria → Labelled target (RNA)



mtrof173	13526
mtrof362	8652
mtrof661	22513
mtrof662	14018
hyaBp	10257
Jpn284	4895
LP20-644	19820
la193	24315
la577	11968

Hybridisation with a single strain #2 Normalisation of results

Reference set #1 - universal pmoA probes
Signal := 100%

Reference option #2 - hyaB probe; externally added DNA

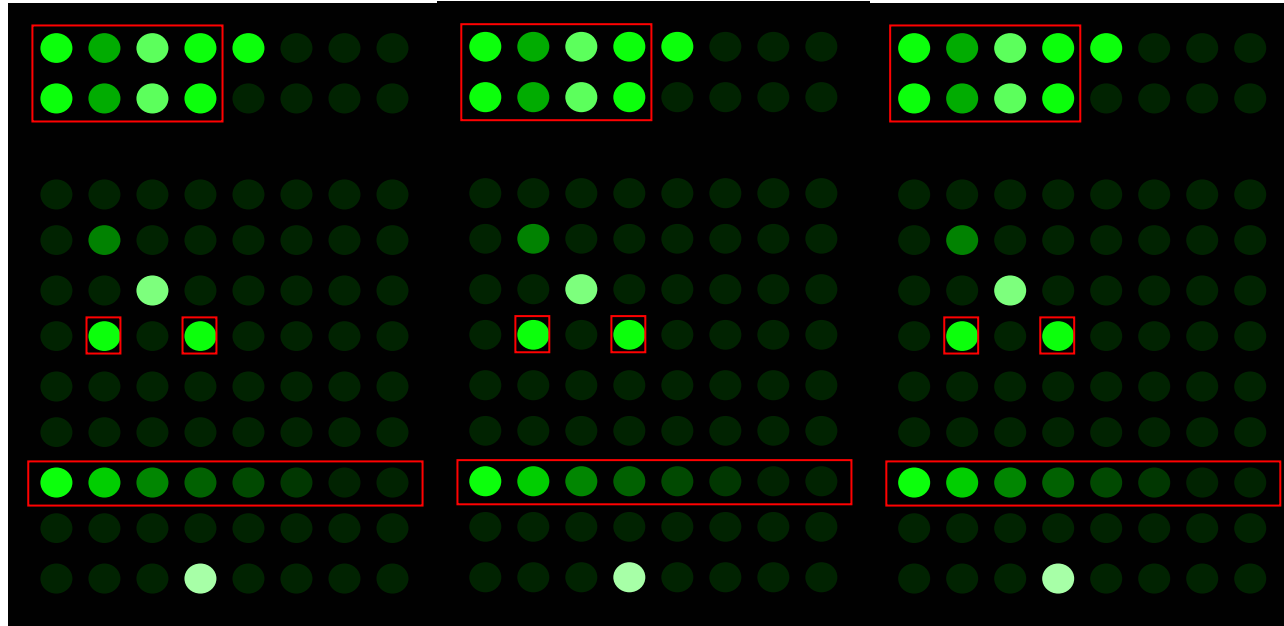
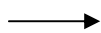


Jpn284	41%
LP20-644	112%
la193	145%
la577	78%

Bacteria

Labelled target (RNA)

Hybridisation with a single strain #3 Parallel arrays on a single chip



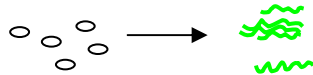
Jpn284	41%
LP20-644	112%
la193	145%
la577	78%

Jpn284	39%
LP20-644	125%
la193	135%
la577	87%

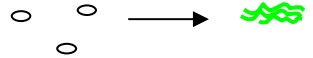
Jpn284	48%
LP20-644	118%
la193	139%
la577	72%

Jpn284	43%
LP20-644	118%
la193	139%
la577	79%

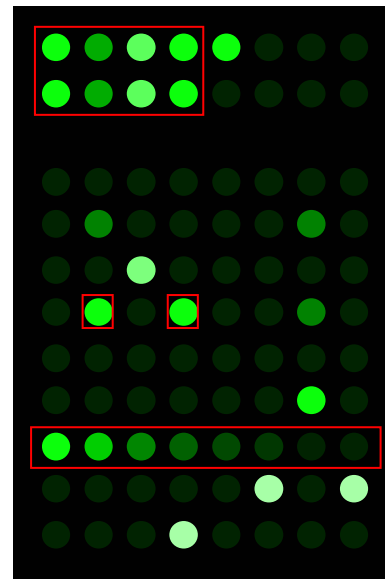
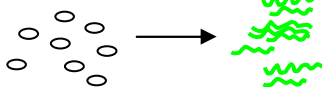
Strain A



Strain B



Strain C



Probe	Max%	Read	Rel.abundance
Jpn284	41%	13%	0.32
LP20-644	112%	41%	0.35
Ia193	145%	52%	0.35
Ia577	78%	28%	0.38
B2-400	72%	18%	0.25
B2all	61%	12%	0.18
Msi270	172%	87%	0.52
II510	228%	114%	0.58
II630	310%	158%	0.49

~30% *Methylobacter* sp. BB5.1

~20% *Methylocapsa acidophila* - related

~50% *Methylosinus sporium* - related

Hybridisation with an environmental mixture

Microbial diagnostic microarrays at Seibersdorf research

- **Techniques**
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 - Bodrossy et al., Environmental Microbiology, July 2003.
- **Pilot array - first high-throughput application**
- **Options to adjust baseline technique**

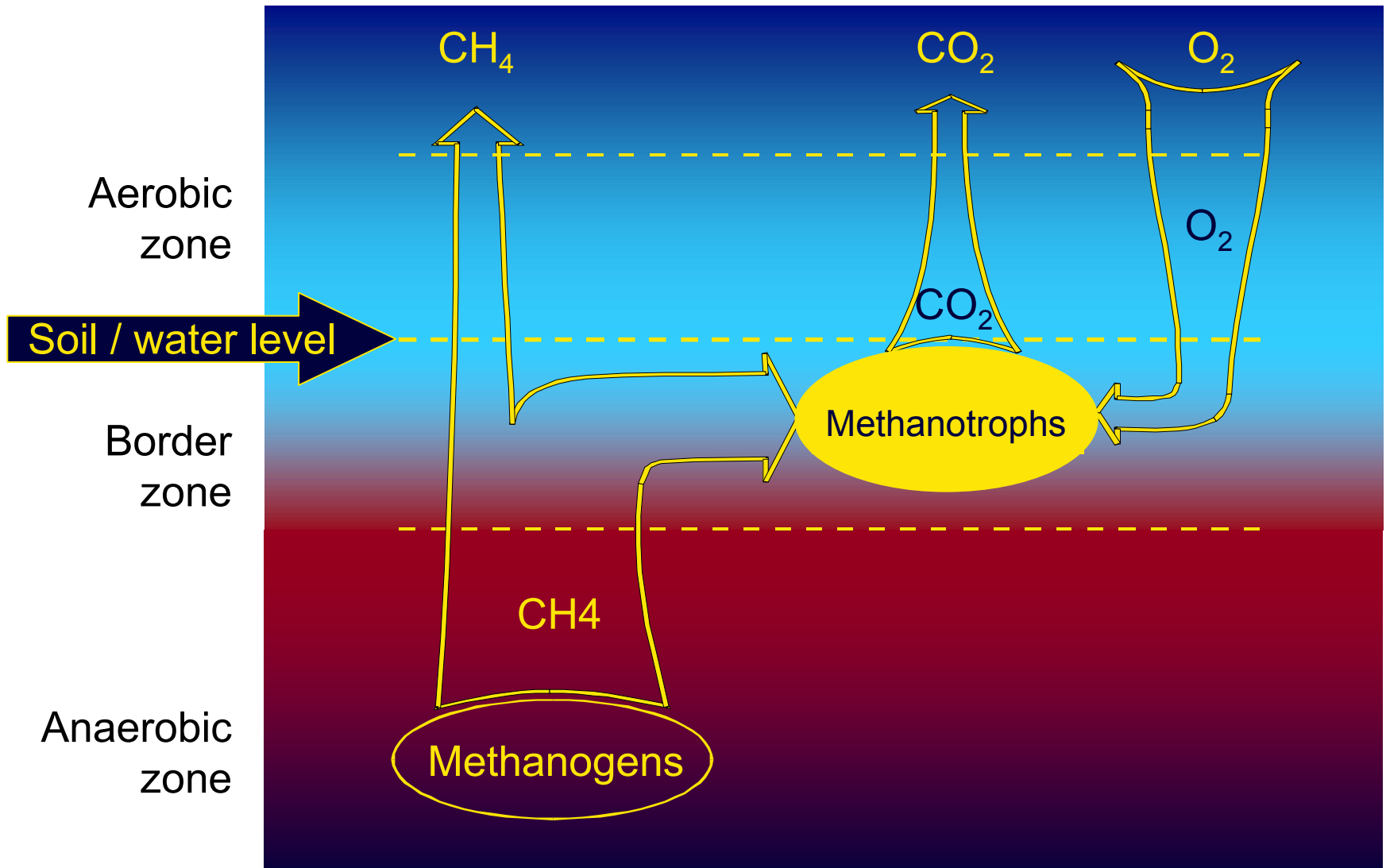
Target group of bacteria for pilot array: Methanotrophs

Methanotrophs are...

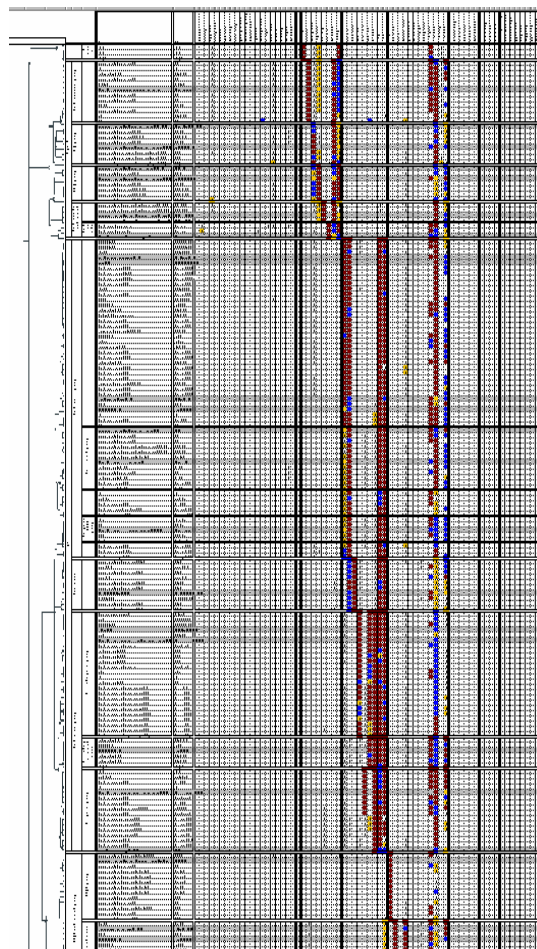
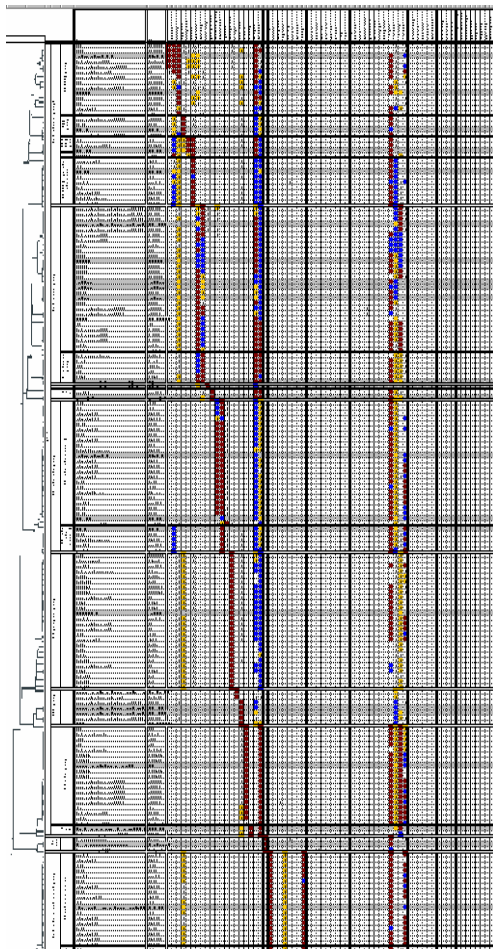
- ... a unique group of bacteria using methane as sole source for carbon and energy
- ... very important in mitigating the greenhouse effect
- ... a taxonomically well defined group of bacteria suiting the requirements for a test group
- ... the *pmoA* gene can be used as a (“functional”) phylogenetic marker (over 500 sequences available)



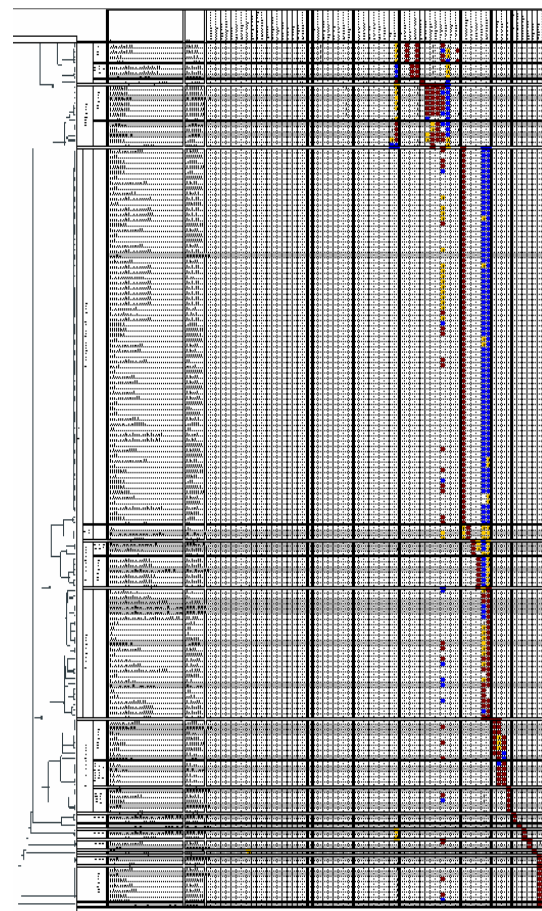
Target group of bacteria for pilot array: Methanotrophs



Final probe set / predicted hyb. behaviour

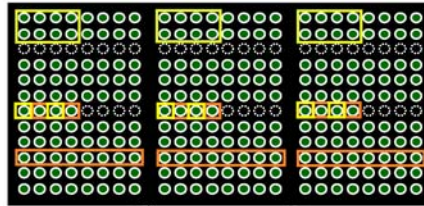


(ARB → CalcOligo → Excel)



Final probe set - layout and validation with pure cultures

(a)

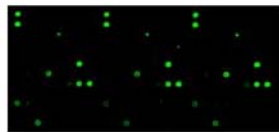


(b)

mtrof173	mtrof362-l	mtrof661	mtrof662-l	Ib559	Mcy262	Mcy409	Peat264
mtrof173	mtrof362-l	mtrof661	mtrof662-l	Msi520	Msi_tri309	Msi270	Msi232
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
Mb460	Mb478	Mb271	511-436	II510	II630	RA14-598	B2rel260
BB51-299	Mb292	Mm275	Mm391	B2-400	B2all343	pmoAMO3-400	xb6-539
PS80-291	peat1-3-287	Mb_SL#1-421	Mb_SL-299	LP21-190	LP21-232	NmNc533	Nsm_eut381
mtrof656	Blank	mtrof656	hyaBp	Blank	Blank	Blank	Blank
Jpn284	Mm_pel467	Est514	LP20-644	PI6-306	PS5-226	NsNv207	NsNv363
Mmb303	Msa661	la193	la577	Nit_rel223	Nit_rel351	Nit_rel470	Nit_rel304
hyaBp 1/2	hyaBp 1/4	hyaBp 1/8	hyaBp 1/16	hyaBp 1/32	hyaBp 1/64	hyaBp 1/128	hyaBp 1/256
Nc_oce426	Mth413	Mc396	501-286	M84P105-451	WC306_54-385	M84P22-514	gp23-454
fw1-639	M90-201	Mcl408	Ib453	MR1-348	gp391	gp2-581	RA21-466

layout

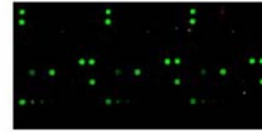
(c)



AOB related clone MeOH12_18



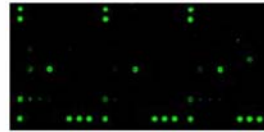
Ms. sporium SE2



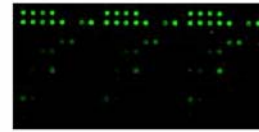
Nm. eutropha



Methylocaldum related clone M90-P75



Clone RA21



Ms. trichosporium OB3b

validation
with pure
cultures

Deviations in the hyb. behaviour - results of the probe set

Unexpected results (expected: PM to 1.5wMM yield positive signal; above 1.5wMM: no signal):

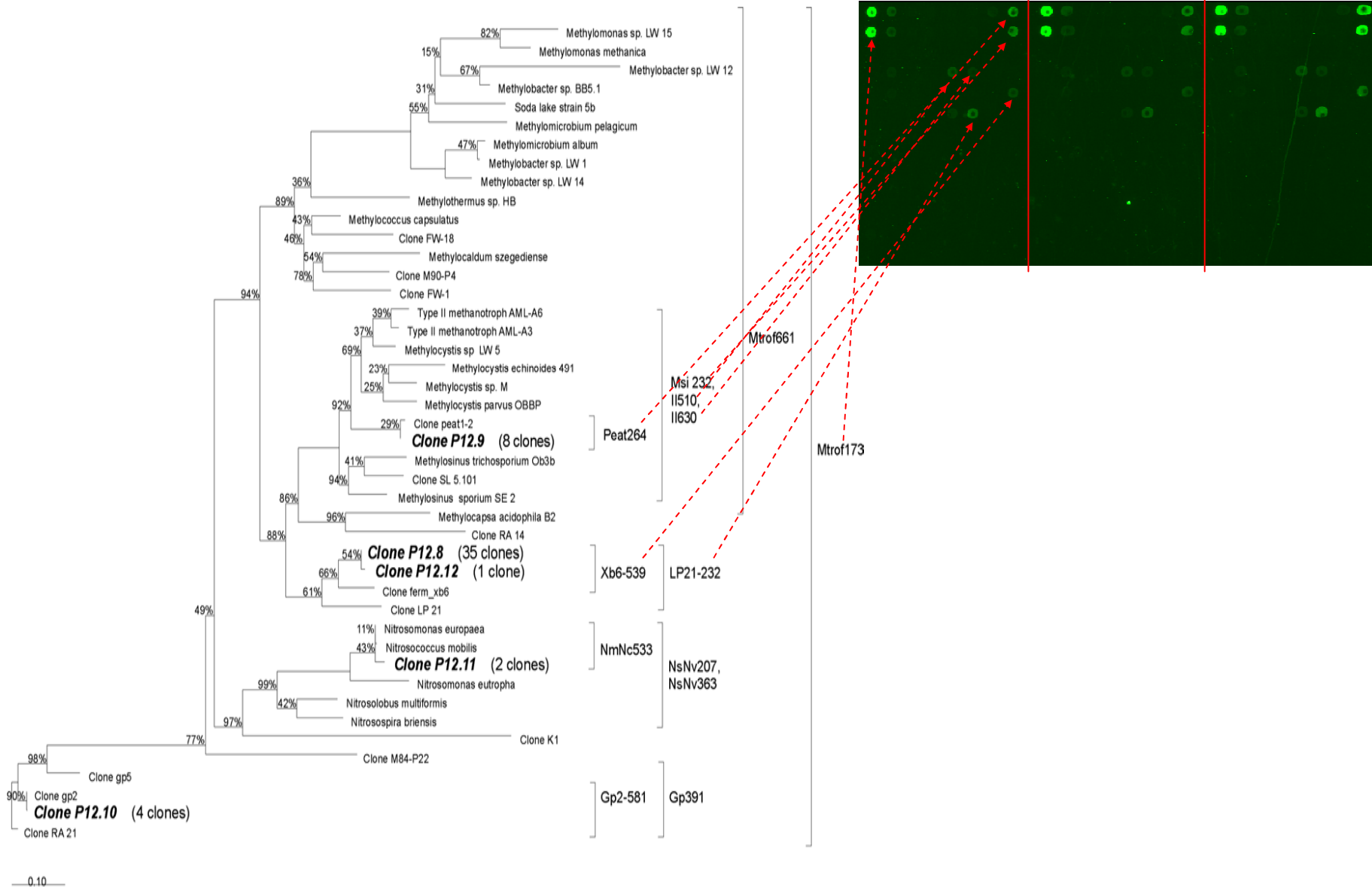
Out of 3660 individual hybridisation reactions (61 validated probes x 60 reference targets):

- 3621 (98.9%) yielded the expected result.
- 39 (1.1%) resulted in false negative or positive signal.
- 17 of the above 39 are associated with long continuous PM regions ($T_m \geq 48$ °C).
- 43 of the 61 probes (70.5%) validated behaved 100% as predicted.

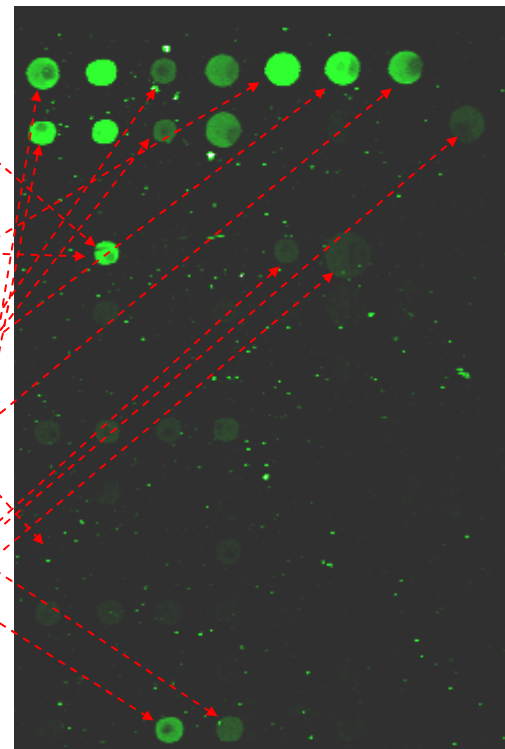
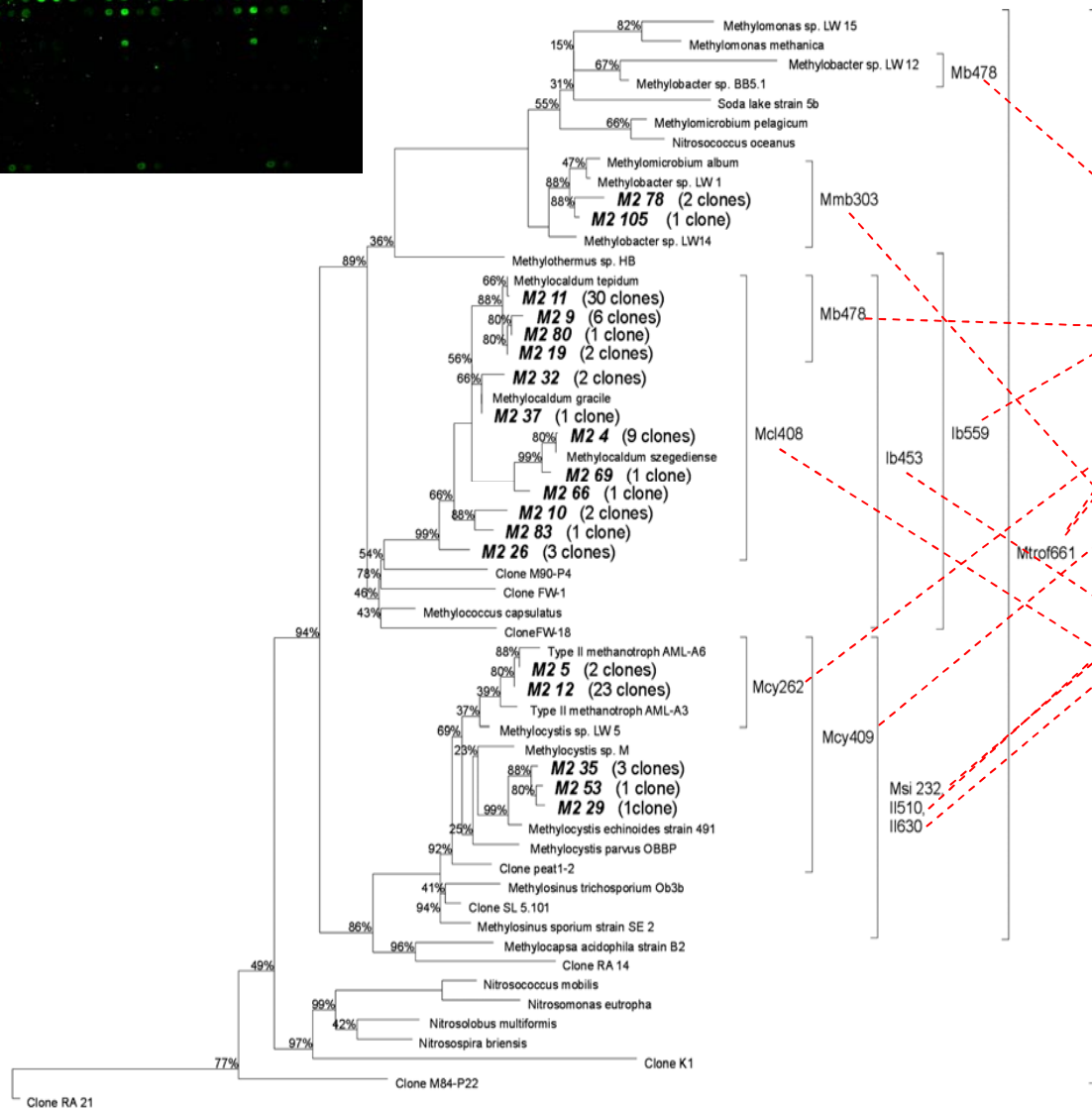
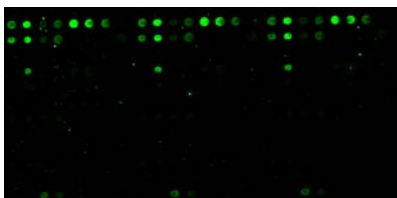
CalcOligo used to weigh mismatches:

- Generates Excel table
- Enables accounting for microarray-specific effects
- Will predict T_m , instead of displaying MM numbers
- Will enable the refinement of the prediction parameters to fit predictions to results

Evaluation with env.samples I: soil microcosm sample



Evaluation with env.samples II: landfill site cover soil



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Full scale application: landfill site experiment



Effect of plant cover on bacterial methane oxidation in the cover soil?

5x: Plant covers:

- Poplar (P+, P-)
- Miscanthus (M+, M-)
- Grass (G+, G-)
- Grass&Alfalfa (GL+, GL-)
- Bulk soil (BS+, BS-)

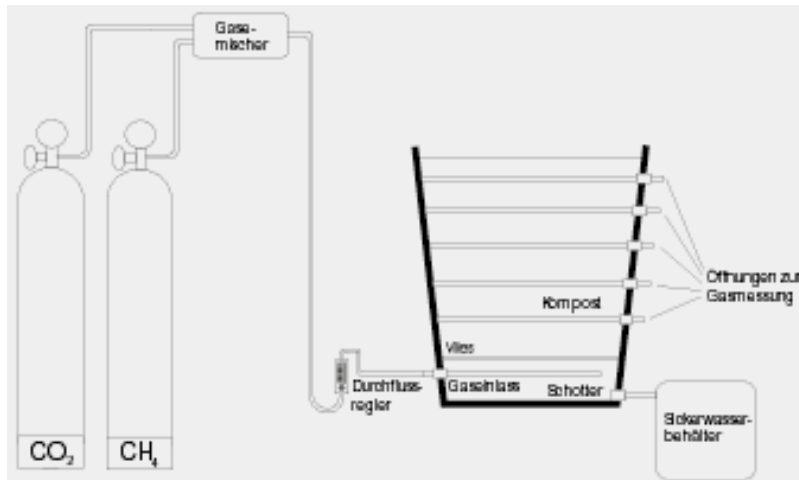
5x depths:

- 10 cm
- 20 cm
- 30 cm
- 50 cm
- 60 cm

• 2x: with/without methane

• 5x: replicates of each pot

250 samples



Full scale application: landfill site experiment

Grass + Alfalfa without methane



Grass + Alfalfa with methane



note: pots' contents are upside down!

← **Anaerobic zone**
 $\text{CH}_4 + \text{CO}_2$

← **Oxidation zone**
 $\text{CH}_4 + 2\text{O}_2 \rightarrow$
 $\text{CO}_2 + 2\text{H}_2\text{O}$

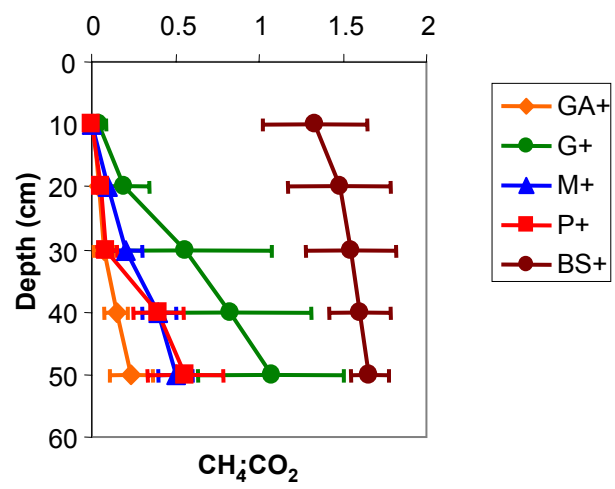
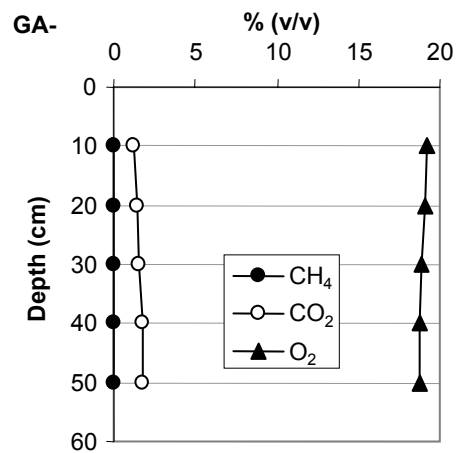
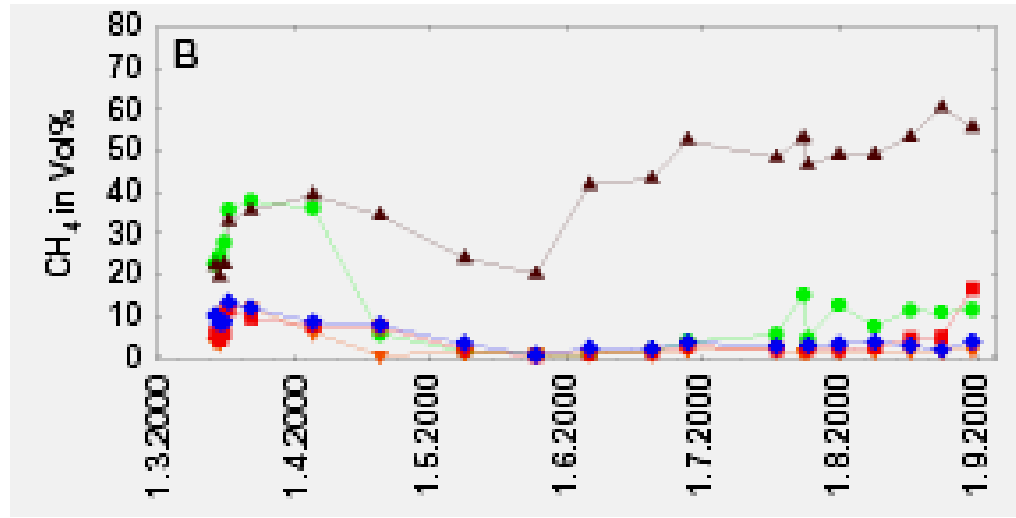
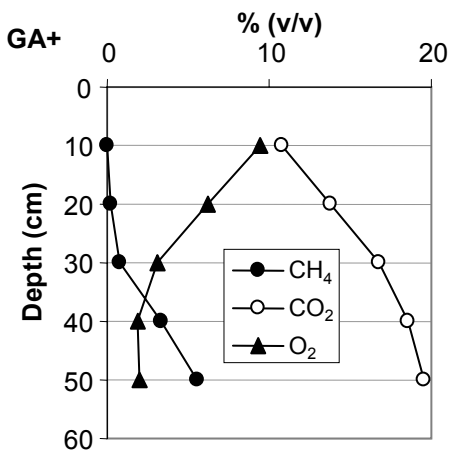
← **Aerobic zone**
 O_2

- Experiment ran for 2 years
- 250 samples out of 50 pots taken
- Soil samples homogenised and analysed using the methanotroph diagnostic microarray

Chemical data also collected:

CH_4 , O_2 , CO_2 , seepage water, %dry matter, %N, %C, C/N, pH, ammonium, nitrate, sulphate, chloride, hydrogen sulphide, position of the oxidation zone

Full scale application: landfill site experiment - oxidation capacities

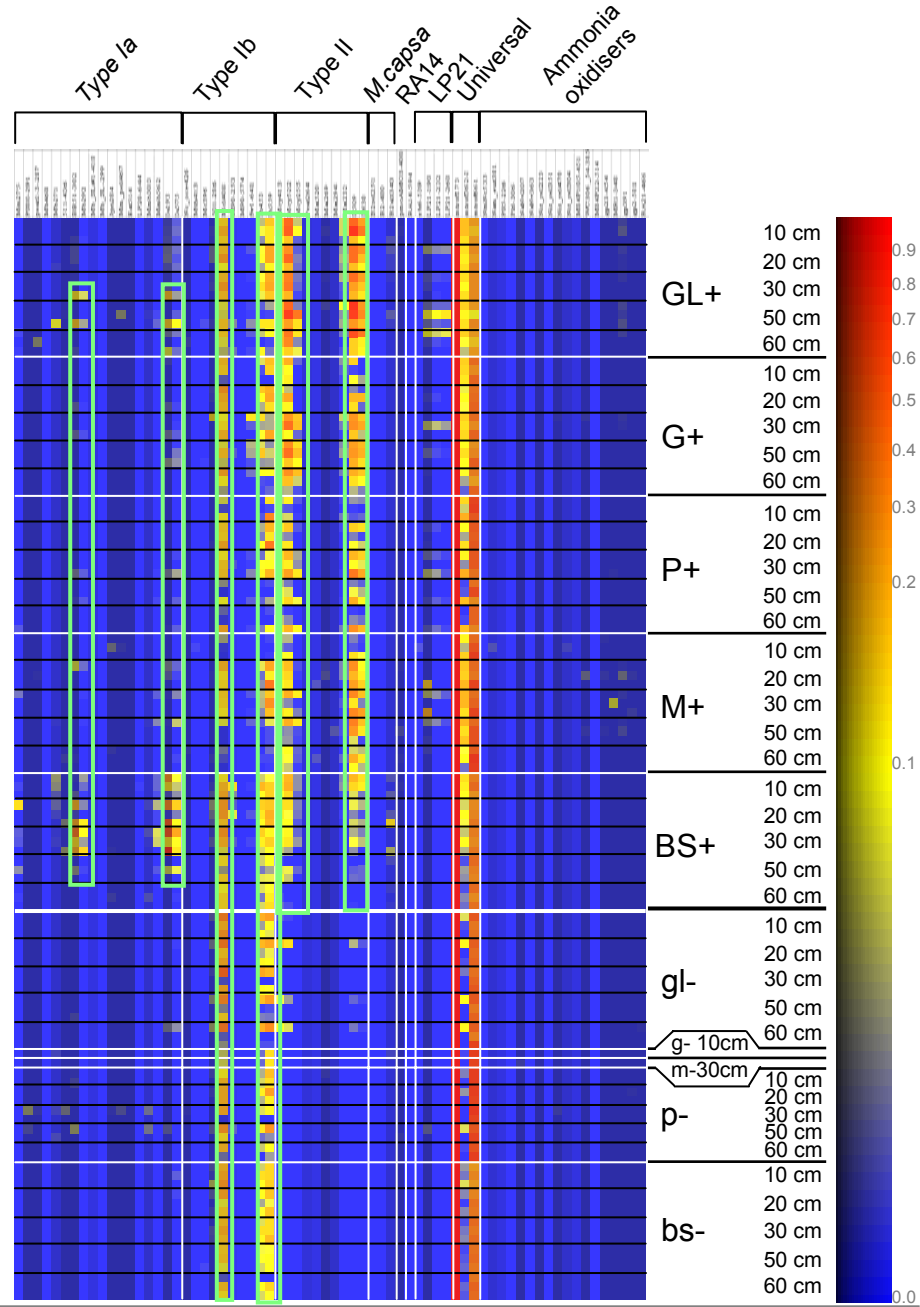
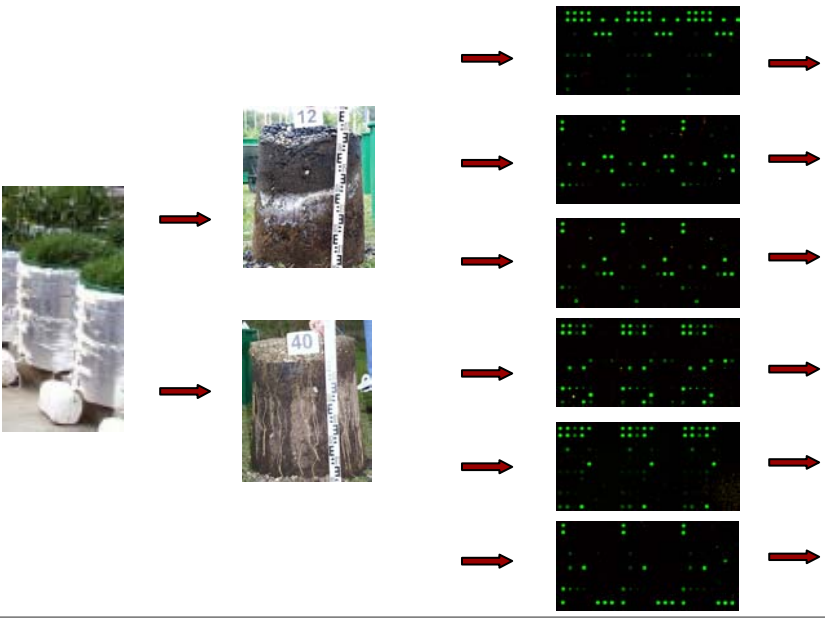


Grass+Alfalfa > Miscanthus, Poplar > Grass >> Bulk soil

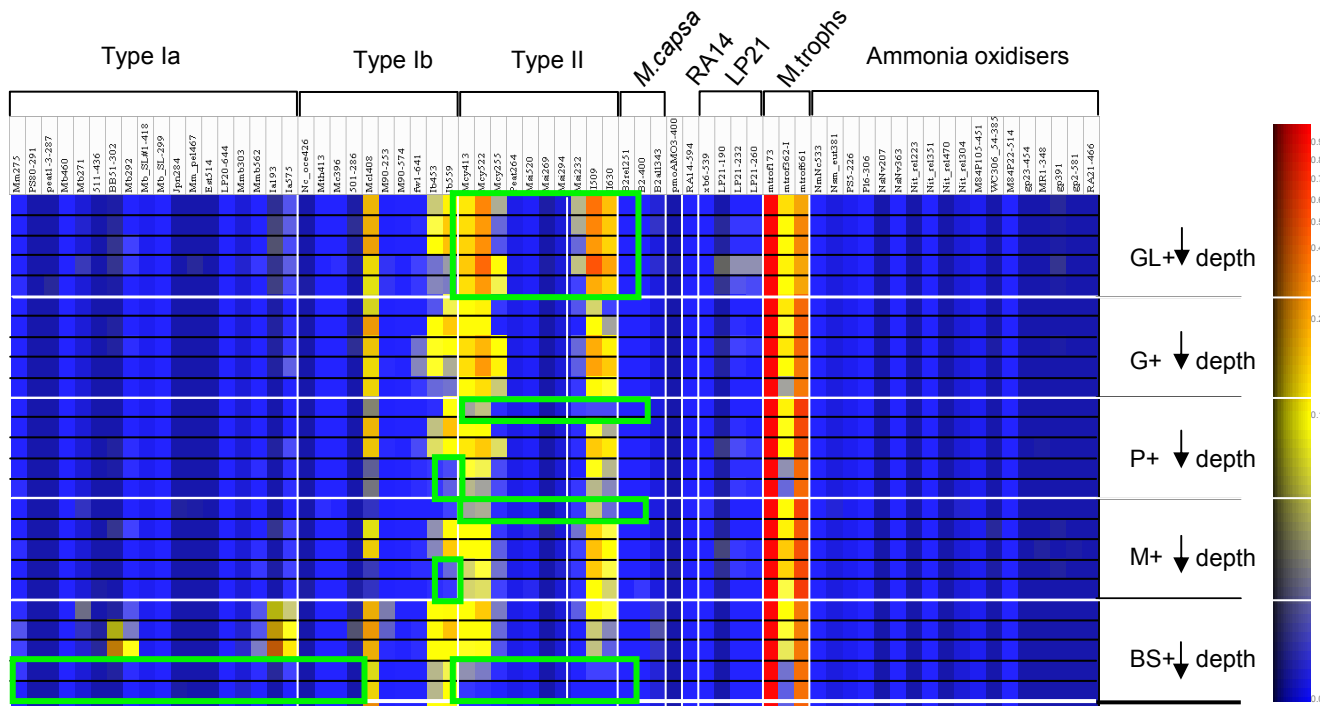
Pilot chip - methanotrophs

Application in landfill site studies

- Type Ib and Type II dominating
- Type II (*Methylocystis*) only if methane added
- Type Ib (*Methylocaldum*) - stable presence
- Type Ia (*Methylobacter*) - in a few pots only
- Sporadic appearance of *Methylomonas*, *Methylochromium*, group LP21 and *Methylocaldum*-related groups
- *Methylococcus*, *Methylosinus* never detected
- (Ammonia oxidisers not targeted by PCR)



Full scale application: parallel samples merged (CH₄ + only)



M.bacter BB5.1
M.bacter BB5.1
and soda lake M.bacter

M.microbium
Type Ia
generic probes

M.caldium
Type Ib
generic probes
All M.cystis
M.cystis group A
M.cystis parvus,
M.cystis echinoides
Type II
generic probes

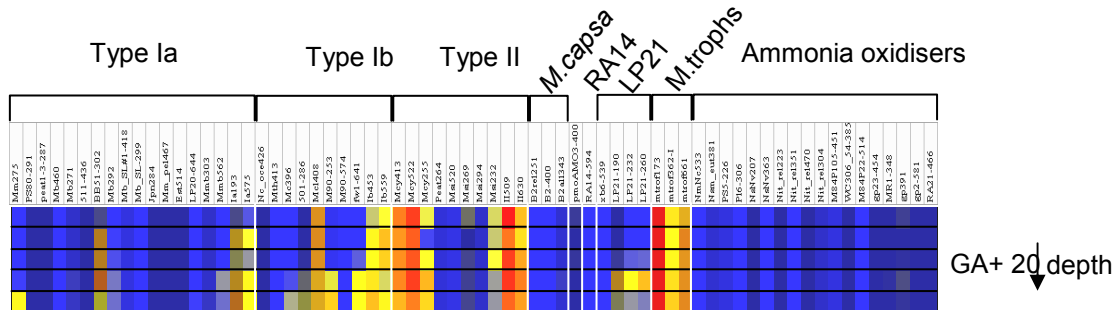
LP21 group
Generic probes
for methanotrophs

Comparing plant covers

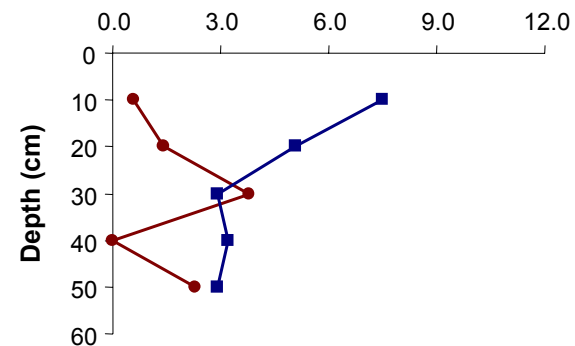
- GA+: highest abundance of type II, throughout the pots
- P+ and M+: low diversity in the top 10 cm
- P+ and M+, 50-60 cm: *Methylocaldum* hardly at all present (resting cells?)
- BS+ below 30 cm: no type II, no type Ia

Full scale application: strange results (outlier pot)

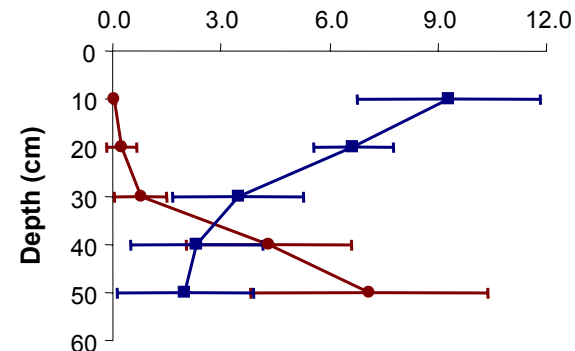
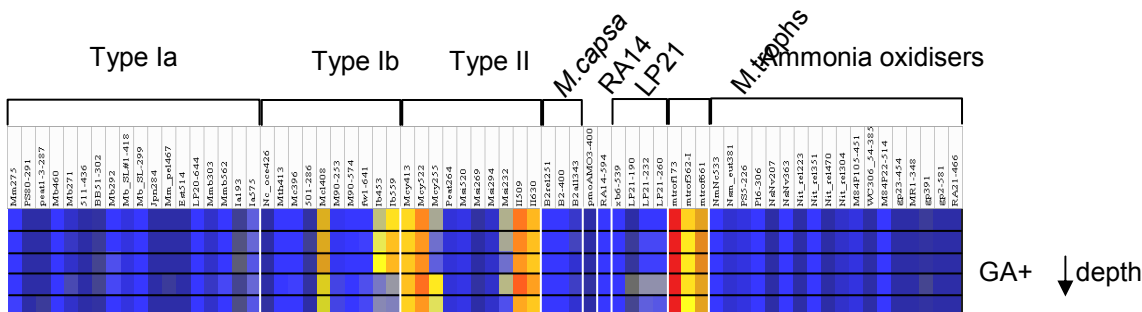
GA+ 20 lysimeter with strange results



Gas profiles: CH_4 , O_2

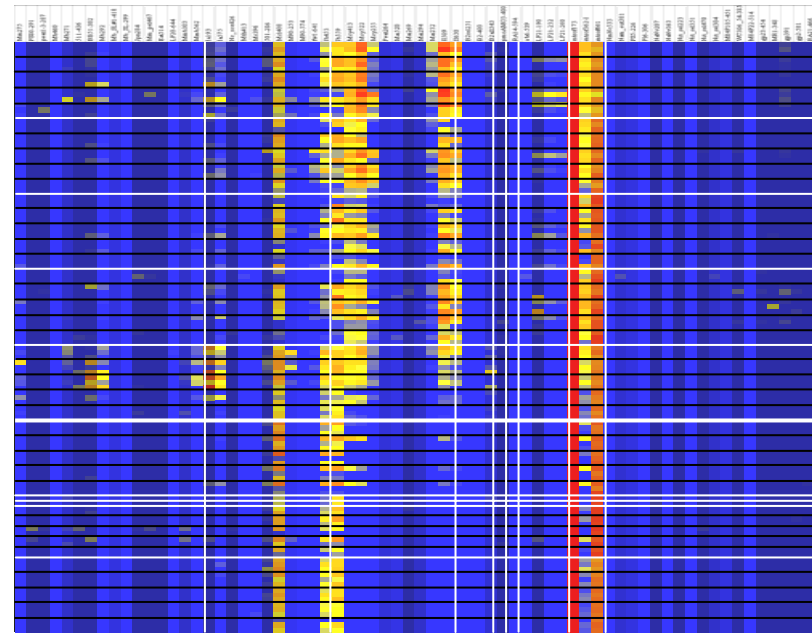


Rest of GA+ lysimeters: average of results



Explanation: air leaked into pot P20 (=> very high diversity)

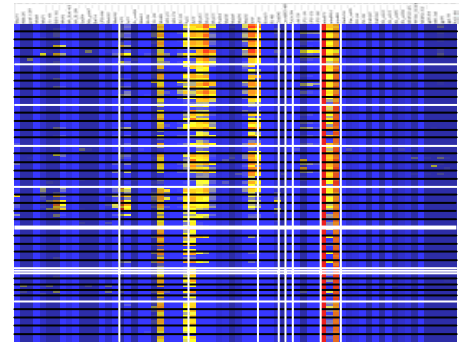
Full scale application: summary



- Key players: *Methylocystis* (type II) and *Methylocaldum* (thermotolerant type I)
- *Methylocaldum* able to survive under low methane conditions as well
- Deeper layers (low oxygen, high methane) have *Methylocystis* species harbouring a second, novel type of pMMO - apparently conferring advantage to the bacteria under these conditions
- Best performance of Grass+Alfalfa plant cover correlates with a high ratio of *Methylocystis* throughout the analysed layers
- Grass+Alfalfa plant cover supports *Methylocapsa*

Pilot array - present potential

- **Aim: Community analysis of a well defined physiological group of bacteria**
- **Criteria: Parallel, high-throughput, high resolution, results comparable on a quantitative basis**
- Up to 1000 oligoprobes applicable - parallel
- Results in 24 hours (starting from samples)
- Resolution down to the species level
- Analysis focused on a physiological group of microbes
- Analysis extended to uncultivated members of this physiological group
- PCR bias known & manageable
- Reliable detection of major populations (bacteria at 5% or more of the total community targeted by the PCR)
- Semi-quantitative results
- Quantitative comparisons, esp. for temporal or spatial changes, possible
- **Generation of high resolution data on community structures**



Microbial diagnostic microarrays at Seibersdorf research

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Challenges – options to meet them

- Improved prediction of the hybridisation behaviour of the oligoprobes
- **Bioinformatics + data on array specific effects (immobilisation, steric hindrance, etc.)**
- Higher resolution needed (below the species level, e.g. medical diagnostics)
- **IGS, *gyrB* and tmRNA databases**
- Phenotype of the detected microbes (pathogenicity, AB resistance, etc.) to be predicted
- **Sequence databases (16S, IGS, *gyrB* with clinical traits linked)**
- Array not focusable to a physiological group
- **16S, 23S (also *gyrB* and *rpoB*) databases**
- PCR not acceptable (too much bias and/or only live bacteria assayed for)
- **Direct detection of rRNA or tmRNA**
- More reliable quantification required
- **2-colour hybridisations**
- Minor groups of bacteria (not dominant ones) need to be reliably detected
- **Sequence specific end labelling of oligonucleotides**
- Shorter time-to-result required (food diagnostics, bedside diagnostics)
- **Direct rRNA/tmRNA detection, evanescent field scanning technology**
- Various limitations in probe design to be overcome
- **LNA residues in DNA oligoprobes**
- Other physiological group of bacteria to be targeted
- **Databases of other genes (*nirS/nirK*, *nifH*, *norB*, *mcrA*, *rbcL*, *ndoB*, etc.)**
- Physiological activities of the bacteria detected to be understood
- **Isotope arrays**

Challenges – options to meet them

Mirzabekov et al., Engelhardt, R

Stahl et al., U.Washington, USA

Wengel et al., U.Southern Denmark, D

IGS, gyrB and tmRNA databases

Rudi et al., Matforsk, N

St Anthony et al., King's College, UK

gyrB with clinical trans links

Kawaguchi et al., GRI, J

Array not focusable to a biological group

16S, 23S (also Wagner et al., U.Vienna, A)

Tiedje et al., Michigan, USA

PCR not acceptable (too much bias and/or only live bacteria)

Direct detection of tmRNA

Zhou et al., Oak Ridge, USA

Chizhikov et al., FDA, USA

More than 1000 paired

DeBellis et al., Segrate, I

2-colour detection systems

Borneman et al., U.California, USA

Cerniglia et al., FDA, USA

Minority species not dominant ones, may not be reliably detected

Sequence specific and labelling of oligonucleotides

Glöckner et al., Max Planck, D

Shorter time (good diagnostics, bedside diagnostics)

Direct rRNA/tmRNA detection technology

Andersen et al., Lawrence Livermore, USA

Various limitations in probe design to be overcome

LNA (Chandler et al., Argonne, USA)

DeRisi et al., U.California, USA

Other physiological activities not detected

Databases of other (Call et al., Washington State Univ., USA)

Ludwig et al., TU Munich, D

Physiological activities of the bacteria detected to be understood

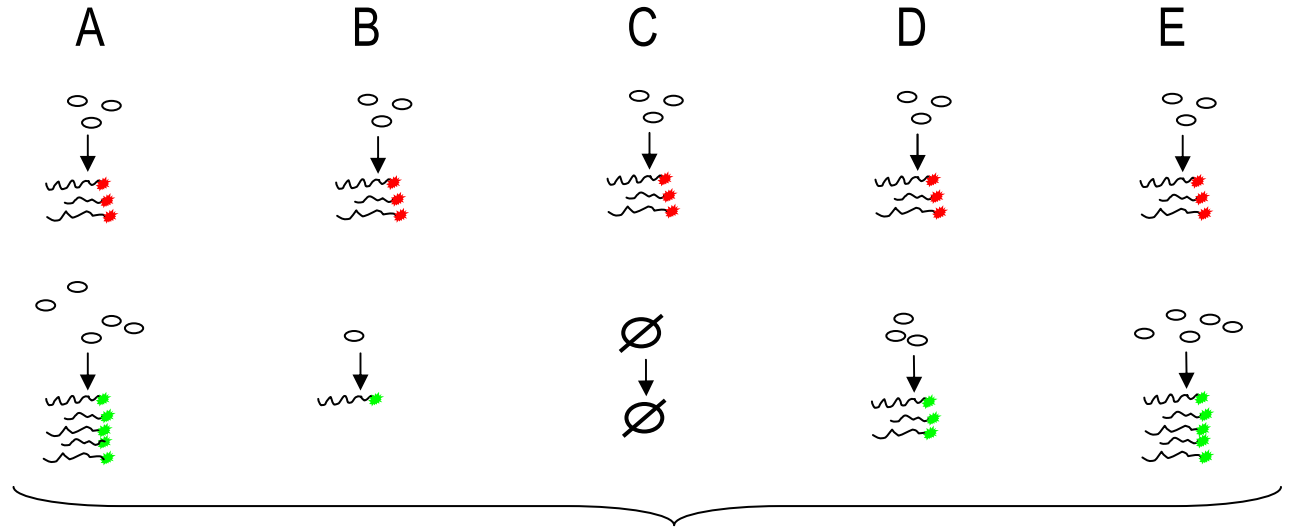
Isotope arrays

Two-colour quantification:

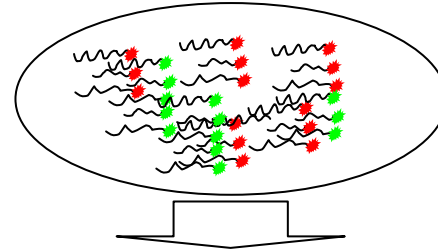
Strains:

Internal control {
Bacteria
Labelled target

Sample {

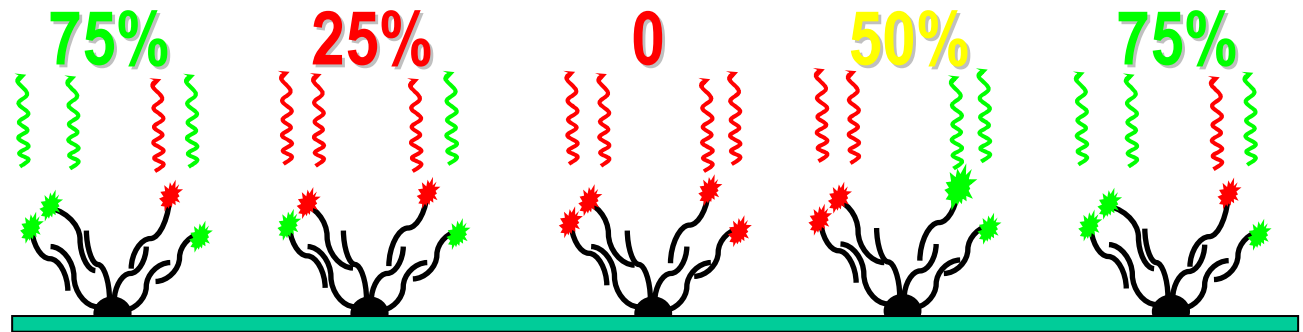


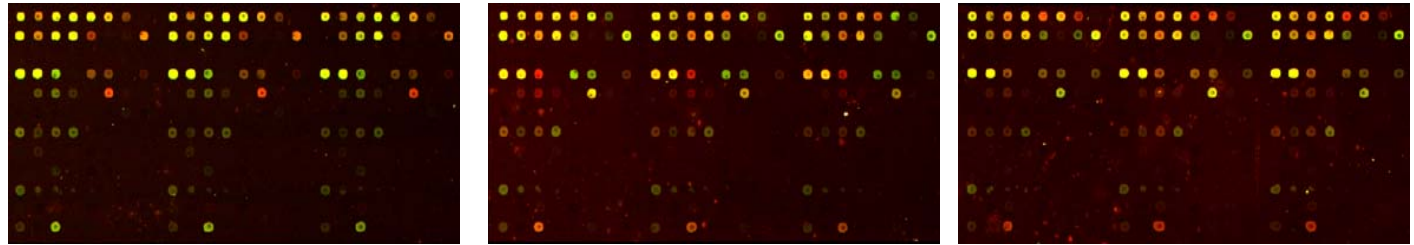
Mixture of labelled target



Hybridisation onto the chip

Chip after hybridisation





Artificial Mixture #1

Artificial Mixture #2

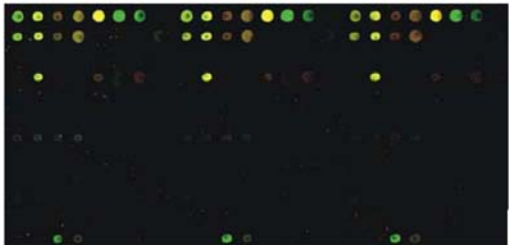
Artificial Mixture #3

	Ratios (%)	Measured ratios (%)	Ratios (%)	Measured ratios (%)	Ratios (%)	Measured ratios (%)
Clone SL-5.102	60	68	1	2.1	10	20
<i>Mcl. szegediense</i> OR2	30	21	3	4.7	1	2.3
Clone SL-5.70	10	6.1	60	36	3	3.6
Clone rbp46	3	4.0	30	43	60	51
Clone JY-6.48	1	1.8	10	12	30	22

Standard deviations are in the range of 0.4-17.2 %

Two-colour quantification: Testing its potential with artificial PCR mixtures

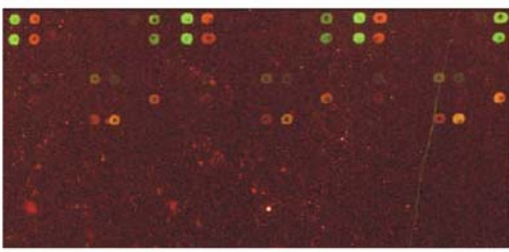
	Probes									
	Mb478	Mcl408	N_lb453	Ib559	Mcy262	Mcy409	Msi232	II510	II630	mtrof173
Max. value	353	82	162	497	238	61	222	55	61	100
1-colour: Cy3 values	149	63	24	234	151	54	14	15	10	100
Procent predicted	42	77	15	47	63	88	6	27	16	100
2-colour: Cy3/Cy5 ratios	48	56	29	61	N.A.	N.A.	56	43	57	100
Procent predicted	24	28	14	30	N.A.	N.A.	28	21	28	100



Landfill site cover soil

A

	Probes						
	Peat264	Msi232	II510	II630	N_xb6-539	LP21-232	mtrof173
Max. value	101	222	17	16	192	306	100
1-colour: Cy3 values	40	18	43	37		12	100
Procent predicted	56	47	48	38		34	100
2-colour: Cy3/Cy5 ratios	51	71	18	21		10	100
Procent predicted	25	35	9	10		5	100



Soil microcosm

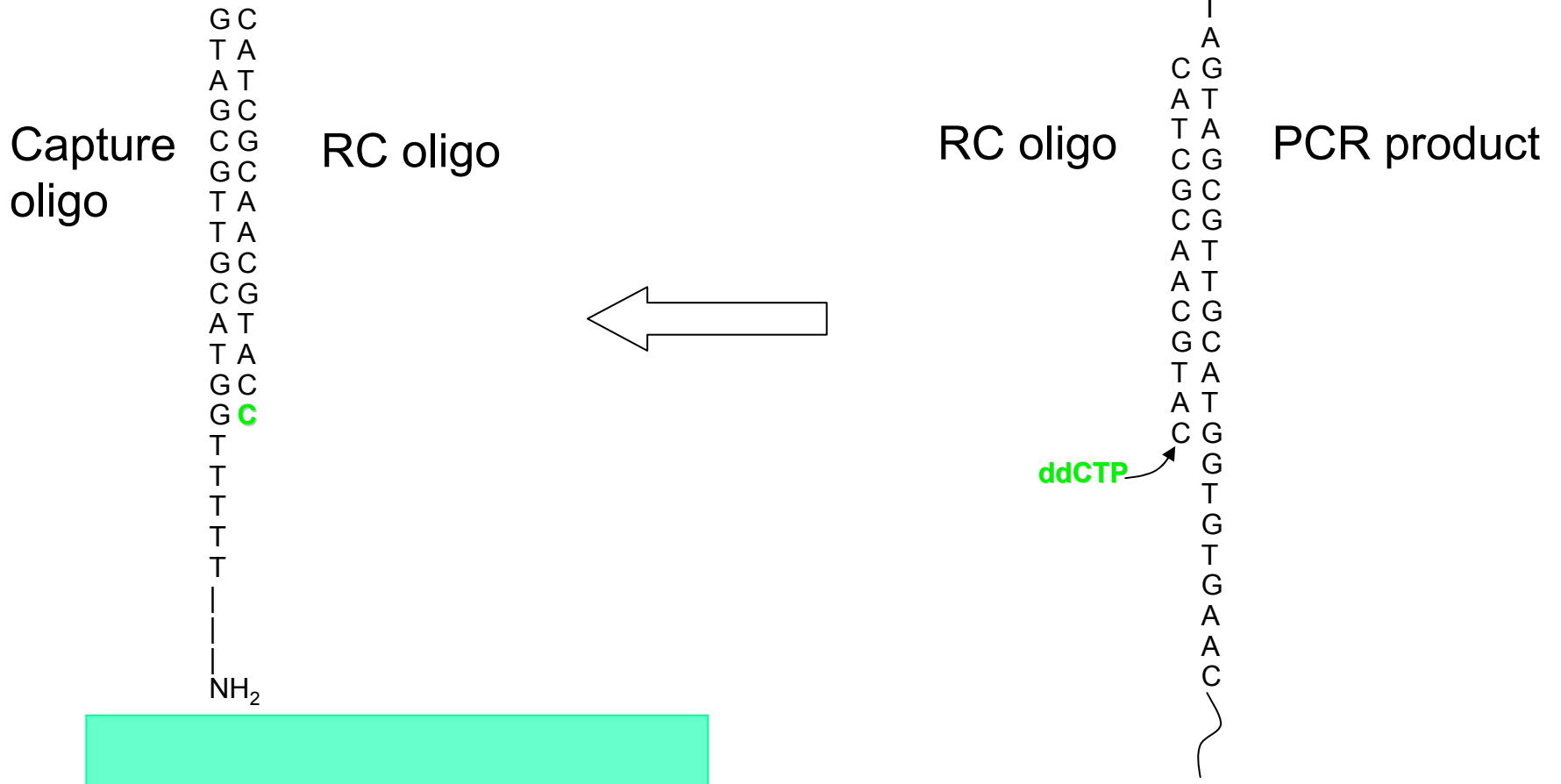
B

Two-colour quantification: Testing against environmental samples

Results are in good agreement with those of independent community analyses.

Sequence specific end labelling of oligos

(Rudi and Holck, NAR, 2003)



Sequence specific end labelling of oligos (Rudi and Holck, NAR, 2003)

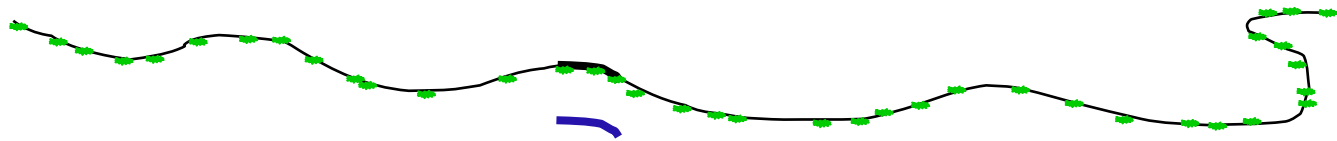
Target:

5' -TGGCATGCACCGTCACGTACGCGCGCCGTTTGCATCGCTACGTCATCGTCAGCTGGCATGCACCGTCACGTACGCGCGCCGTTTGCATCGCTACGTCATCGTCAGC -3'
 3' -ACCGTACGTGGCAGTGCATGCGCGCGGGAAACGTAGCGATGCAGTAGCAGACGACCGTACGTGGCAGTGCATGCGCGCGGCAAACGTAGCGATGCAGTAGCAGACG -5'

Synthesized, labelled target:

5' -TGGCATGCACCGTCACGTACGCGCGCCGTTTGCATCGCTACGTCATCGTCAGCTGGCATGCACCGTCACGTACGCGCGCCGTTTGCATCGCTACGTCATCGTCAGC -3'

Probe on the array: 3' -AAACGTAGCGATGCAGTAGCAG-5'



Target:

5' -TGGCATGCACCGTCACGTACGCGCGCCGTTTGCATCGCTACGTCATCGTCAGCTGGCATGCACCGTCACGTACGCGCGCCGTTTGCATCGCTACGTCATCGTCAGC -3'
 3' -ACCGTACGTGGCAGTGCATGCGCGCGGGAAACGTAGCGATGCAGTAGCAGACGACCGTACGTGGCAGTGCATGCGCGCGGCAAACGTAGCGATGCAGTAGCAGACG -5'

Synthesized, labelled target:

5' -TTTGCATCGCTACGTCATCGTC -3'

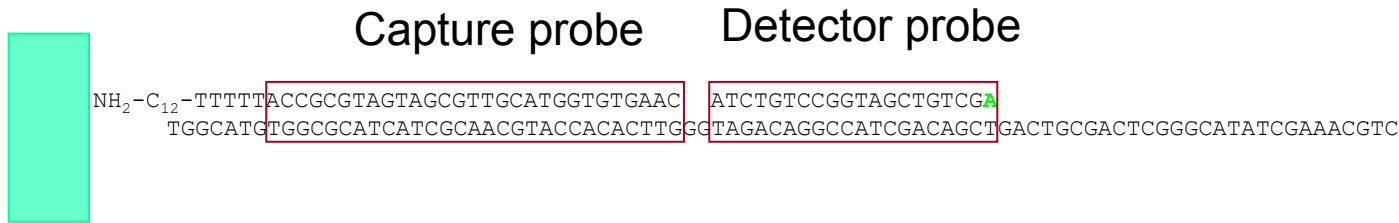
Probe on the array: 3' -AAACGTAGCGATGCAGTAGCAG-5'



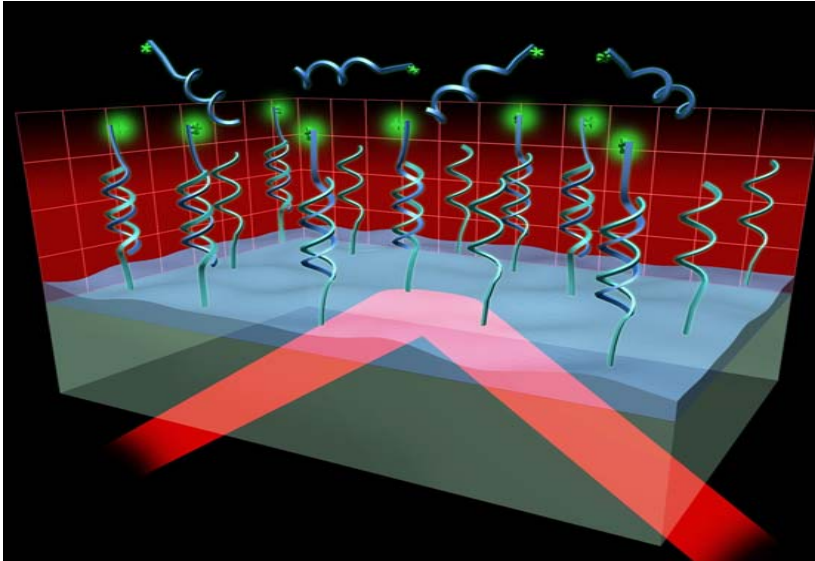
Direct detection of RNA (rRNA, tmRNA)

Requires naturally amplified nucleic acids - rRNA: 10^3 - 10^5 ; tmRNA: 10^2 - 10^3 copies per cell

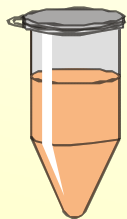
- Direct labelling - commercially available kits (Micromax ASAP, LabelIt, etc.)
- Polyacrilamide microarray pads combined with direct labelling (originally developed by Andrei Mirzabekov, now applied and improved by Dave Stahl's group, Univ. Washington)
- Capture + (labelled) detector probes (Chandler *et al.*, 2003)



DNA Biosensorchips for real-time hybridization analysis (Evanescent field technology)



- evanescent field excitation of bound fluorophors
- sensor chips with integrated flow channel
- separation/washing free detection of hybridization
- **association** and **dissociation** kinetics

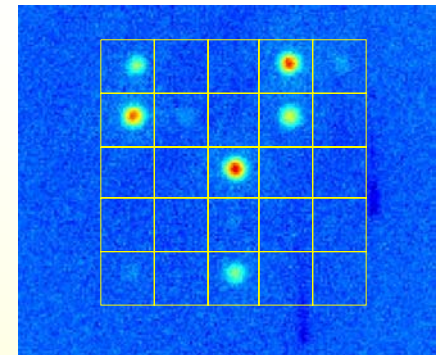


Cy5-labelled PCR product



sensor chip

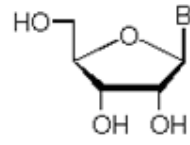
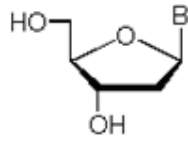
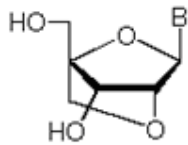
3 min



results (slope)

LNA oligos

Exiqon A/S



LNA - Locked Nucleic Acid

DNA

RNA

- **Short diagnostic regions - increase T_m**

- **LNA residues at diagnostic positions - increase specificity**

- **Potential to increase T_m of the whole probe set - improve S/N, increase sensitivity**

Type	Duplexes	T_m (°C)	ΔT_m (°C)/mod.
DNA:DNA	5'-tga gct ggt ggg-3' 3'-act cga cca ccc-5'	35.0	-
LNA:DNA	5'-tGa GcT gGt Ggg-3' 3'-act cga cca ccc-5'	52.5	+3.5
DNA:RNA	5'-gtg ata tgc-3' 3'-cac uau acg-5'	28	-
LNA:RNA	5'-gTg aTa Tgc-3' 3'-cac uau acg-5'	50	+7.3

T_m 's of duplexes	Perfect match 3'-acgaccac	Single mismatch 3'-acg g ccac	ΔT_m
5'-TGCTGGTG	71°C	45°C	26°C
5'-tgctggtg	35°C	25°C	10°C

Summary:

- A comprehensive set of methods and protocols for microbial diagnostic microarrays - adaptable to a wide range of upcoming novel technology platforms (evanescent field based detection, bead arrays, capillary bead arrays, cantilevers, electronic detection, etc.)
- High resolution, semi-quantitative picture of the bacterial community obtainable from any samples / environments
- Looking for strategic partnership and/or contract research to develop microbial diagnostic microarrays
- Please visit our websites: www.diagnostic-arrays.com ==> www.arcs.ac.at

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