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Validation of a more sensitive method for using spotted oligonucleotide DNA microarrays for functional genomics studies on bacterial communities

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Summary

Spotted oligonucleotide microarrays potentially offer a wide scope of applications for microbial ecology, especially as they improve the flexibility of design and the specificity of detection compared to PCR product based microarrays. Sensitivity, however, was expected to be problematic, as studies with the more sensitive PCR-based cDNA microarrays indicate that only genes from populations contributing to more than 5% of the community DNA can be detected. We evaluated several parameters to increase sensitivity and then tested applicability for bacterial functional genomics. The optimal parameters were the use of 5′-C6-amino-modified 70-mers printed on CMT-GAPS II substrates at a 40 μM concentration combined with the use of Tyramide Signal Amplification labelling. This protocol allowed detection of single copy genes belonging to an organism contributing to 1% or more of the total community. To demonstrate its application, we detected the specific aromatic oxygenase genes in a soil community degrading polychlorinated biphenyls (PCBs). This increase in sensitivity is important if oligonucleotide microarrays are to be used for simultaneous monitoring of a range of functions performed by different microorganisms in the environment.

Introduction

Many of nature's metabolic processes are sustained by mixed communities rather than by populations of a single microorganism. This applies to a wide range of environments, e.g. natural soils and sediments, pristine (Bowman *et al*., 2000) or subject to human interference (Nogales *et al*., 1999), water treatment reactors (Lee *et al*., 1999; Pelz *et al*., 1999) and animal oral (Whittaker *et al*., 1996) and intestinal ecosystems (Santo Domingo *et al*., 1998). Microbial ecologists have focused mostly on unravelling the composition and structure of microbial communities (Amann *et al*., 1995; Liu *et al*., 1997; Zhou *et al*., 2002). However, because of limitations of the experimental tools to mine the community's diverse metabolic capacity, little is known about mechanisms of community functioning, dynamics or inter- and intracommunity interactions.

Because of the challenges posed by the vast genetic diversity in the microbial world, the use of microarray technology in microbial ecology has been proposed (Cho and Tiedje, 2001; Liu *et al*., 2001; Murray *et al*., 2001; Wu *et al*., 2002) with the prospect to eventually enable simultaneous monitoring of multiple members of a community ('community-on-a-chip'). To determine the community composition, microarrays composed of phylogenetic markers (e.g. 16S rRNA) have been developed (Liu *et al*., 2001; Koizumi *et al*., 2002; Wilson *et al*., 2002). Array techniques using random DNA fragments or specific PCR-amplified genes have been developed as an alternative approach for species identification and community structure analysis (Cho and Tiedje, 2001; Jaccoud *et al*., 2001; Murray *et al*., 2001). Also, PCR-based cDNA microarrays have been reported to detect selected genes in the environment (Wu *et al*., 2002).

Compared to the PCR-based platform, spotted oligonucleotide microarrays, using synthesized oligonucleotides as probes, provide an enhanced flexibility in design, independent of the culturability of the targeted organisms, enabling the design of probes for specific regions of a gene while avoiding the more conserved domains. Consequently, they can offer a higher level of hybridization specificity (Hughes *et al*., 2001; Ye *et al*., 2001). The use of one 50–70 nucleotides long probe per gene has been successfully implemented for comparative expression profiling (Stingley *et al*., 2000; Talaat *et al*., 2002). When interested in complex communities of bacteria, a crucial factor concerning microarrays is their limited sensitivity. Microarrays with 700 bp PCR products as probes were

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unable to detect populations contributing to less than 5% of the total community DNA (Cho and Tiedje, 2002). The reduced probe length of oligonucleotides results in a loss in sensitivity when all other parameters are the same (Denef, 2001; Ye *et al*., 2001). Although the methodological optimization, specifically regarding sensitivity issues, when using spotted oligonucleotide arrays has been performed for expression profiling, SNP genotyping and minisequencing (Kane *et al*., 2000; Lindroos *et al*., 2001; Relógio *et al*., 2002), no attempt has been made to examine this technology's capabilities to detect or quantify community gene composition.

We evaluated the applicability of spotted oligonucleotide microarrays to detect specific aromatic oxygenases that catalyse key steps in the biodegradation of polychlorinated biphenyls (Robertson and Hansen, 2001). We systematically optimized spotted oligonucleotide microarrays when using genomic DNA as the sample by comparing several microarray fabrication parameters (substrate chemistry and modification, length, and concentration of spotting of the oligonucleotide probes) and labelling strategies (direct Cy-dNTP incorporation, amino-allyl labelling and post-hybridization signal amplification). Using the optimized protocol, we determined the detection limit and examined if our method could be applied to detect selected genes in sediment microcosms (J.L.M. Rodrigues, C.A. Kachel, M.R. Aiello, J.F. Quensen, O.V. Maltseva, T.V. Tsoi and J.M. Tiedje, in prep.).

Results

Microarray fabrication optimization

To determine which microarray substrate delivers the most sensitive and consistent results, hybridization signals and the background levels on six commercially available microarray substrates were compared (Fig. 1). Superaldehyde substrates produced the highest signal-to-noise ratio with the greatest reproducibility between the replicates (Fig. 1A). When comparing the mean local background of all probes on the different substrates, the lowest and most consistent background levels were observed when using CMT-GAPS II substrates (Fig. 1B). Based on the results of signal intensity and background noise, we selected Superaldehyde and CMT-GAPS II substrates for subsequent investigation. Although higher signal intensities were observed when using one of the other tested substrates (substrate X3), preference was given to the substrates that gave most consistent results.

Variation in (i) length (ii) modification and (iii) concentration of spotting of the probes for each gene, enabled us to investigate the influence of these factors on the sensitivity of microarray hybridizations. A more detailed look at the Cy5 signals per probe category on CMT-GAPS

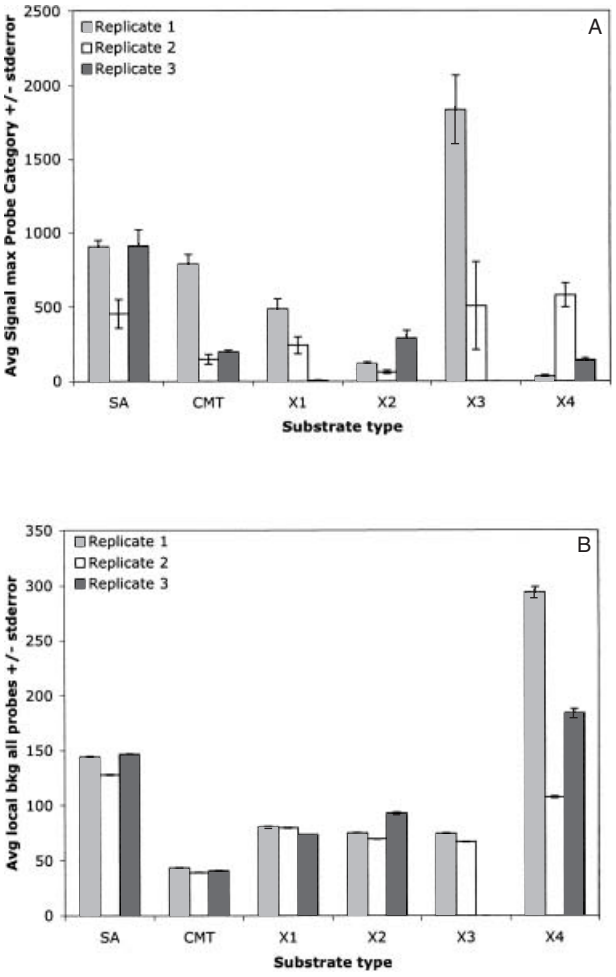

Fig. 1. Comparison of microarray substrates. Labelling reactions and hybridizations were performed in triplicate using direct Cy5-labelling of the PCR product mixture.
A. For each of the tested substrates, the mean 635 nm signals (Cy5) of all probes of the probe category with the highest signal to noise ratio (= optimal probe category) for each of the replicates were compared. Error bars indicate the standard error between the probe signals for the different genes in one replicate.
B. The average of the local background of all probes for each of the replicates. Error bars indicate the standard error between local background of all probes on each of the replicate arrays. X1 = poly L-lysine (CelAssociates), X2 = epoxysilane (CelAssociates), X3 = EZ-Rays (Apogent Discoveries), X4 = 3D-Link (Motorola).

II (Fig. 2A) and Superaldehyde substrates (Fig. 2B), indicated that on both substrates 70-mers produced up to two times higher signals than did 45-mers, indicating that longer oligonucleotide probes resulted in improved sensitivity. The use of amino-modification increased Superaldehyde signals by an average of 1.7-fold, whereas the only significant effect on CMT-GAPS II signals was the disappearance of negative density effects (signal saturation/reduction) at higher concentrations of spotting. For CMT-GAPS II substrates, signals increased up to a 40 μM concentration for unmodified probes whereas the increase was maintained up to 60 μM for modified probes. For

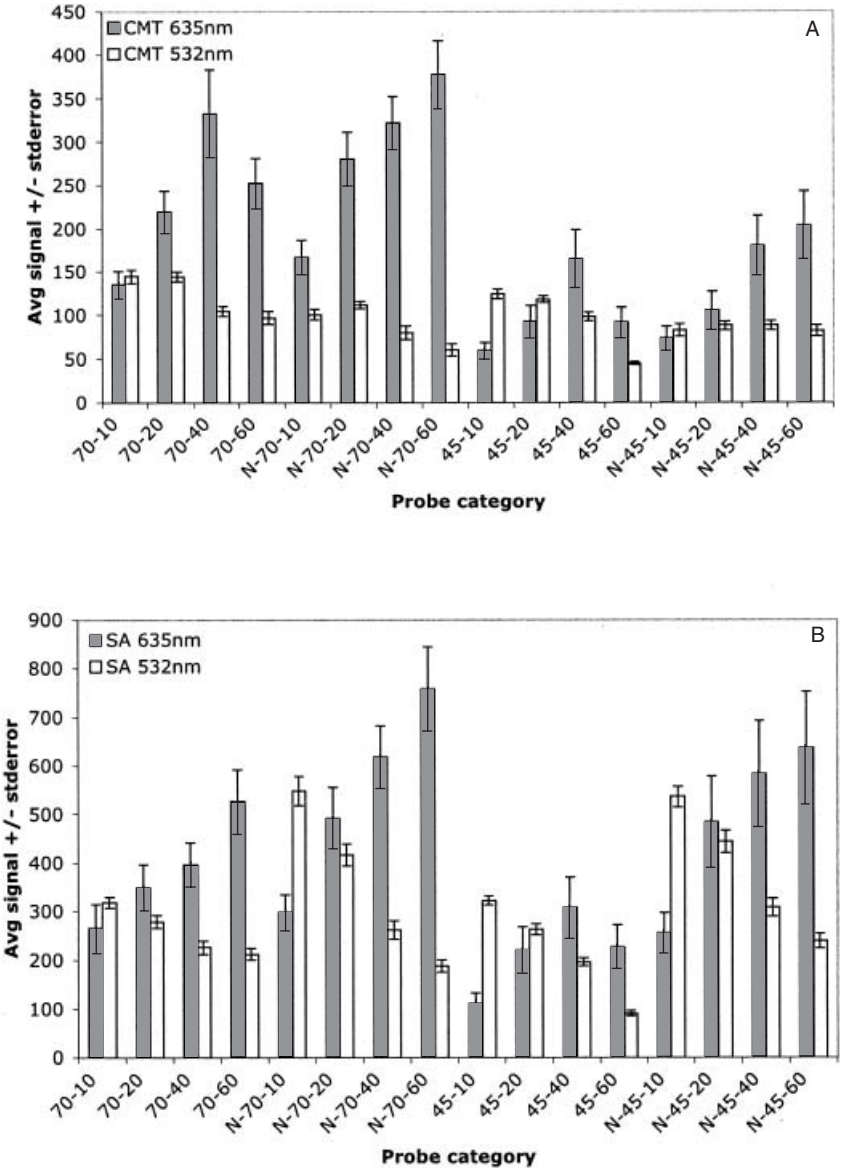

Fig. 2. Comparison of probe length, modification and concentration of spotting on the selected substrates. Signal intensities after local background substraction of all probe categories of (A) CMT-GAPS II and (B) Superaldehyde substrates from experiments in Fig. 1. Mean Cy5 signals (black) and Cy3 signals (grey) of all probes were sorted per category (length, modification, spotting concentration) and standard error between the three experimental replicates was calculated. Probe category codes indicate [modification(N if modification)-length-concentration].

Superaldehyde substrates, the increase of signal was observed up to 60 μM for all probes.

Interestingly, the Cy3 signals, produced by the lambda internal control, especially those on the Superaldehyde substrates (Fig. 2B), revealed a negative linear correlation (modified: $y = -113x + 650$, $R^2 = 0.98$; unmodified: $y = -56x + 380$, $R^2 = 0.99$) between total spotting concentration (bacterial/yeast probe + internal control probe) and signal intensity produced by the hybridized lambda-Cy3 target. Based on this indication of negative effects of probe density, we decreased the concentration of the internal control to 10 μM for further studies and decided not to use the lambda internal control for technical normalization (Cho and Tiedje, 2002), due to the bias this would cause to the data.

Labelling influence on sensitivity

To further improve the sensitivity, direct, amino-allyl and TSA labelling were compared (Fig. 3). A similar signal-to-noise ratio was attained for amino-allyl labelling and direct incorporation, using Superaldehyde substrates. A significant increase in signal intensities was obtained however, when using TSA. To maintain low background levels for the latter, CMT-GAPS II substrates were used.

The lowest variation between replicate labellings and hybridizations was attained when using direct incorporation, when normalizing the amount of incorporated fluorophores between the replicates before hybridization. Because of inconsistencies in background levels for amino-allyl labelling, not reflected in Fig. 3, we cannot

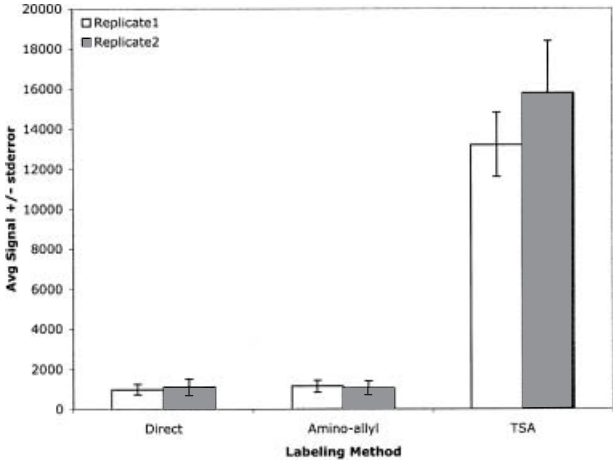

Fig. 3. Comparison of labelling methods. Average 635 nm (Cy5) signals after local background subtraction of the optimal probe category, i.e. the modified 70-mers spotted at 40 μM for CMT-GAPS II and 60 μM for Superaldehyde substrates, when labelling and hybridizing a genomic DNA mixture using three different labelling methods. Two replicates per labelling method were carried out. Direct incorporation and amino-allyl labelling hybridizations used Superaldehyde substrates and were scanned at a PMT of 600, whereas TSA was carried out on CMT-GAPS II substrates and scanned at a PMT of 450 [saturation (signals > 65000) at PMT of 600]. Error bars indicate the standard error between all probes of the optimal probe category of the respective replicate, thus representing the variation between the signals of the different probes (genes) on one microarray slide. Labelled target concentrations were 14.4 ± 0.2 ng DNA μl^{-1} (0.57 ± 0.02 pmoles Cy5 μl^{-1}) for direct, 55.1 ± 5.5 ng DNA μl^{-1} (3.33 ± 0.0 pmoles Cy5 μl^{-1}) for amino-allyl and 81 ± 0.0 ng DNA μl^{-1} for TSA labelling hybridizations.

conclude that the same reproducibility was attained as for direct incorporation. Relative to the increased signal intensity, the TSA system produced a low slide-to-slide variability. The variation for the TSA system originated only from the hybridization and the signal amplification steps as hybridization mixtures for both replicate slides were split from the same biotin-dCTP labelling. The influence of length and concentration differences on TSA signals was similar as observed for direct incorporation, whereas little influence of modification was observed (Fig. 4).

Verification of improved detection limits

To measure the sensitivity and assess the applicability of the optimized protocol to track the targeted genes in a mixed community, we prepared artificial community DNA samples by mixing bacterial gDNAs with mouse DNA in a range of ratios, simulating a community where the bacteria of interest would contribute 1–10% of a total of 10^8 cells (Table 2). To allow slide-to-slide comparison, we normalized signals on each microarray slide using the signals of the Ste3 control probe, for which a PCR product was spiked in equal amounts into every labelling reaction. For each of the studied genes, a linear relationship existed between the signal intensities produced and the amount of starting material used (Fig. 5). The slopes varied in steepness depending on the gene. The multicopy (10 copies/cell) genes *ohbB* and *fcbB* presented steeper slopes (average = 1.535) than those for the single-copy *bph* genes (average = 0.616). The intercept with the *X*-axis, i.e. the detection limit, of the average trend line for the single-copy genes was 0.96% of the total community, while it was 0.54% for the multicopy genes. Therefore, to enable detection of all targeted genes, the population of the organism containing these genes has to comprise 1% or more of the community.

Detection of biodegradative genes in soil

To determine whether our optimized method for spotted oligonucleotide microarrays was sensitive enough to spe-

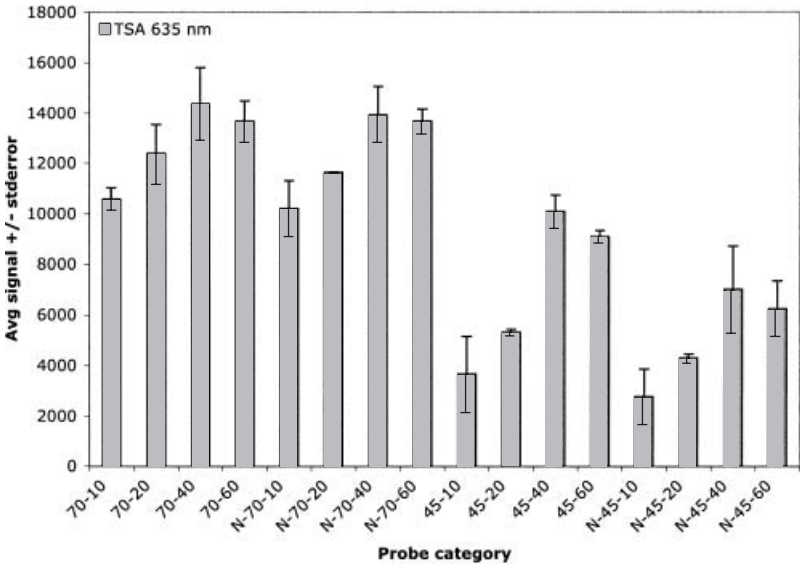

Fig. 4. Signal intensities of all probe categories of the TSA hybridizations. Mean 635 nm (Cy5) signals of all probes, summarized in Fig. 3, sorted per category (length, modification, spotting concentration) with standard error between the two experimental replicates are shown. Probe category codes indicate [modification(N if modified)-length-concentration].

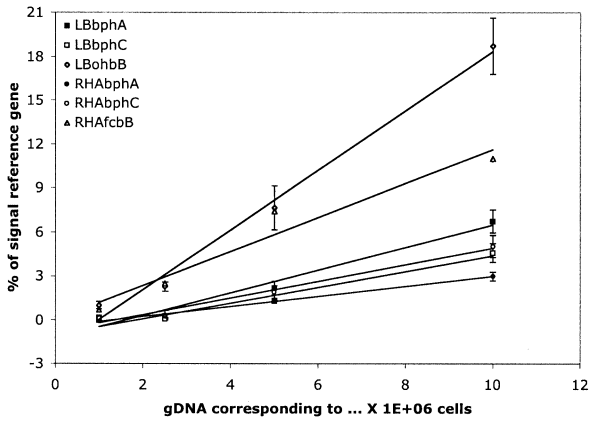

Fig. 5. Sensitivity analysis. We used the optimized method to label and hybridize a range of artificial community DNA samples, composed of bacterial gDNAs mixed with mouse DNA, simulating DNA extracted from 10^6 to 10^7 targeted cells of a total of 10^8 cells. The mean signal intensities (635 nm) with standard error between replicates (on the same slide and between slides) of all probes of the modified and unmodified 70-mers at 40 μM concentration, scanned at a PMT voltage of 480. Two replicate experiments were performed with 1% and 10% targeted cells, while only one for was carried out for the intermediate data points. Each data point on figures A and B included data of two replicate spots per array, per gene, per probe category. The data presented as percentage signal relative to the corresponding Ste3 control (modified or unmodified, and per replicate array on the same slide). Data sorted per organism and gene.

cifically detect genes in environmental samples, we used community DNA from our sediment microcosm studies containing the native population (4.82×10^8 cells g^{-1} by direct count) and inoculated with 10^4 cells g^{-1} of the PCB-growing recombinant organisms LB400(pRO41) and RHA1(pRHD34) (J.L.M. Rodrigues, C.A. Kachel, M.R. Aiello, J.F. Quensen, O.V. Maltseva, T.V. Tsoi and J.M. Tiedje, in prep.). The PCBs in the sediment were degraded during the incubation period and the populations of LB400 grew to 2.00 ($\pm$0.02) $\times 10^7$ cells g^{-1} and RHA1 to 4.95 ($\pm$0.52) $\times 10^5$ cells g^{-1}, as measured by real-time PCR, at their respective sample times of 10 and 5 days. These quantitative data were further supported by plate counts on selective media for the two recombinant strains, 1.42 ($\pm$0.33) $\times 10^7$ cells g^{-1} and 9.60 ($\pm$0.96) $\times 10^5$ cells g^{-1} respectively. Duplicate labellings and hybridizations were performed for each of the DNA samples using the optimized protocol (i.e. Tyramid Signal Amplification using CMT-GAPS II substrates, with 70-mers printed at 40 μM, modified or unmodified). This allowed us to detect the plasmid-located multicopy genes for both organisms (*ohbB* and *fcbB*) and the single-copy genes of LB400 (*bph*A and *bph*C). No signal was detected for the single-copy genes of RHA1 (Fig. 6). No signal was detected for any of the probes targeting genes not present in the community DNA sample (yeast genes, RHA1(pRHD34) genes in LB400(pRO41) sample and vice versa) indicating that the signals were specific.

Discussion

Because specificity, quantification and especially sensitivity are critical issues to how applicable DNA microarrays can be to microbial ecology studies, we systematically optimized spotted oligonucleotide microarray methodology to maximize sensitivity for this application. Given the current spotted microarray technology and labelling techniques, we have arguably reached the highest sensitivity, while preserving consistency of the detected hybridization signals. The most significant improvements were due to TSA labelling, which is based on post-hybridization signal amplification. The optimal method used TSA in combination with CMT-GAPS II substrates printed on with 70-mer oligonucleotides at a concentration of 40 μM. 5′-C6-Amino-modification of the oligonucleotides had minor influence on the sensitivity.

This protocol not only compensated the loss in sensitivity due to shorter probes, but improved it approximately five times beyond the detection limit for conventional cDNA microarrays (Cho *et al*., 2002). The latter study used dilutions of PCR products of the targeted genes in a non-specific carrier (mouse genomic DNA) and calculated that the organism containing the targeted gene has to contribute to at least 5% of the community DNA for the gene to be detectable. In another report, which included sensitivity studies of cDNA microarrays, Wu *et al*. (2002) reported detection of *nirS* genes in 1 ng of pure genomic DNA and in 25 ng of soil community DNA. However, no

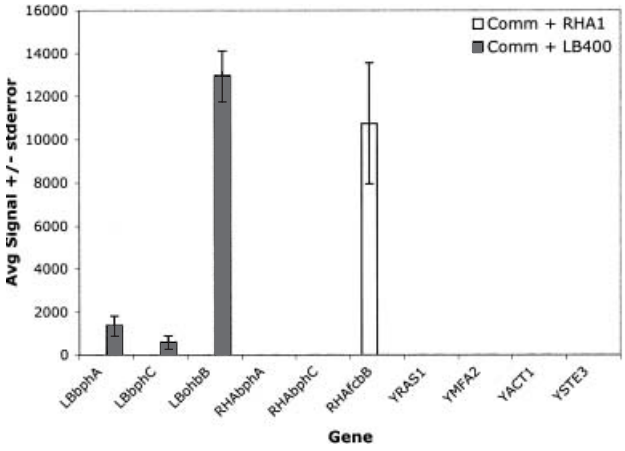

Fig. 6. Detection of genes in sediment DNA. The optimized protocol was used to label 200 ng community DNA extracted from sediment microcosms. Average of the absolute 635 nm (Cy5) signals (after background subtraction) of the optimal probe categories after hybridization of labelled soil microcosm community DNA, using the TSA system, scanned at a PMT voltage of 600. Communities consisted of both indigenous populations and inoculated LB400(pRO41) (black) or RHA1(pRHD34) (grey). Error bars indicate standard error between the signals of the replicates. Labeled target concentrations were 24.0 $\pm$ 0.3 ng DNA μl^{-1} for LB400(pRO41) microcosm hybridizations and 29.9 $\pm$ 2.3 ng DNA μl^{-1} for RHA1(pRHD34) microcosm hybridizations.

dilution into carrier DNA was carried out nor was it known how much of the community DNA was contributed by organisms containing the targeted *nirS* gene. Competition during labelling and subsequent hybridization is a factor of major importance when assessing sensitivity. When using PCR-amplified copies of the targeted genes, a significant decrease in signal was observed when diluting these products in mouse gDNA as compared to using the same amounts of PCR products without dilution (data not shown). Besides other sensitivity-determining factors, such as target secondary structure and steric hindrance, the detection limit is thus dependent on the ratio of the targeted sequence over the total DNA in the sample, rather than on absolute amounts of the targeted sequence, which in large part is due to the characteristics of the enzymatic labelling. Higher amounts of starting material result in a lower amplification rate by the enzyme during labelling (Bioprime Labeling Kit documentation, Invitrogen), leading to a lower number of labelled fragments containing the specific sequence targeted by the oligonucleotide probe. This characteristic was supported by results by Talaat *et al*. (2002) who showed that higher amounts of gDNA starting material led to a lower number of probes producing signal.

We also determined the sensitivity limit of our optimized method for detecting functional genes in an artificial community, composed of a mixture of genomic DNA from different organisms. We found, as did Cho *et al*. (2002) and Wu *et al*. (2002), a linear correlation between the amounts of the genes present in the sample and the detected signal, affirming that a quantitative assessment of functional genes in microbial communities is possible. However, the characteristics of this linear relationship, i.e. slope and intercept, were dependent on the target gene (Fig. 5). The higher than expected signals for the *ohbB* gene can be explained by our recent discovery of a highly similar gene (>95%) in the LB400 genome, the labelled target of which also hybridized to the *ohbB* probe. We did note a positive correlation between the guanidine percentage of the probe and the slope of the sensitivity trend lines. Although our probe set is too small to be conclusive, it could indicate that sequence dependency of hybridization signals originates during hybridization. We cannot exclude, though, that bias is introduced during target preparation, i.e. during labelling. In either case, sequence composition influences on hybridization signals are important when attempting to make quantitative conclusions based on microarray hybridization data. Based on our results, current probe design software is incapable of avoiding this drawback. This puts a limit on the use of this method for high-throughput quantitative detection, unless standard curves are determined for a representative subset of the probes. Alternatively, the use of multiple probes per gene, averaging the influence of probe sequence on the hybridization signal, or a microarray platform that allows flexible probe design adjustments could resolve this dependency issue. Preliminary data showed that genomic DNA fragmentation may reduce the bias in labelling (data not shown).

This study differs from previous microarray method developments for studying microbial communities (Cho and Tiedje, 2002; Wu *et al*., 2002) in the use of oligonucleotide probes as compared to long PCR products (cDNA microarrays). Spotted oligonucleotide microarrays offer a significant increase in probe sequence fidelity and only sequence information is needed for their synthesis, avoiding problems with culturability of the species of interest and allowing synthesis of probes with consensus sequences, targeting a group of closely related genes. Additionally, the use of the relatively short and single-strand oligonucleotide probes results in a reduction of complexity at the probe level, which improves hybridization performance and consistency. There are limitations to the use of oligonucleotide probes for microbial ecology such as the reduced sensitivity due to probe length, and the fact that the existing gene sequence database is probably only a very small fraction of the extant sequences in nature.

Although the hybridization signal generally increased with increasing concentrations, negative effects of density took over at higher probe densities as clearly seen in the Cy3 signals, produced by the lambda internal control (Fig. 2B). While the lambda probe concentration was unaltered (20 μM in each spot), the Cy3 signals unexpectedly decreased with increasing total probe concentration (bacterial or yeast probes + lambda internal control probe), We suggest that this negative effect originated from steric or electrostatic hindrance limiting the availability for hybridization or causing increased competition on the probe level.

In our initial studies, we were unable to detect any hybridization signals using PCR-based microarrays, when labelling 100–500 ng microbial community DNA extracted from the same sediment microcosms by direct incorporation of Cy-dCTP (Denef, 2001). Our optimized method was now able to specifically detect the targeted genes in the same community DNA samples. Based on total cell counts and selective plate counts, these samples contained $3.0 \pm 0.7\%$ LB400(pRO41) and $0.2 \pm 0.02\%$ RHA1(pRHD34), whereas the real-time PCR data indicated a presence of $4.15 \pm 0.05\%$ LB400(pRO41) and $0.1 \pm 0.01\%$ RHA1(pRHD34). The results confirmed the expected detection of all targeted LB400(pRO41) genes, while the positive results for the RHA1(pRHD34) sample were slightly better than expected based on the results from the sensitivity study on the artificial community DNA samples (Fig. 6). These specific signals, observed at the

fcbB probes, could be explained by a higher plasmid copy number than assumed, both in the artificial community construction and the RTm-PCR data. The array results showed that our strategy provides a significant improvement in the applicability of microarrays over currently used methods for environmental bacterial genomics studies.

Experimental procedures

Bacterial strains, plasmids, media and DNA preparation

Bacterial strains and plasmids used in this study are listed in Table 1. The PCB-degrading organisms were *Burkholderia* sp. LB400 (Bopp, 1986) and *Rhodococcus* sp. RHA1 (Seto *et al*., 1995) and their genetically modified derivatives RHA1(pRHD34) containing the *fcbABC* operon for 4-chlorobenzoate (4-CBA) dechlorination (Rodrigues *et al*., 2001) and LB400(pRO41) containing the *ohbRABC* operon for ortho-CBA dechlorination (Tsoi *et al*., 1999). Wild-type strains were routinely grown at 30°C in mineral medium K1 (Zaitsev and Karasevich, 1985), supplemented with biphenyl (1 g l^{-1}). The recombinant strains RHA1(pRHD34) and LB400(pRO41) were grown at 30°C in mineral medium K1 supplemented with 4-CBA (2 mM) and 2-CBA (2 mM), respectively. Genomic DNA was isolated as described earlier (Sambrook and Russell, 2001). Plasmid DNA was isolated using the Wizard® SV96 Plasmid Purification System (Promega).

Oligonucleotide probes

We constructed a microarray containing modified (5′-acrydite™ (EZ-Rays), 5′-C6-amino (other substrates) and unmodified 45-mer and 70-mer probes (IDT, Coralville, IA) for the PCB-degradative genes *bphA1* and *bphC* of LB400 and *bphA* and *bphC* of RHA1 and the chlorobenzoate-degradative genes *ohbB* and *fcbB* (Table 1). Positive and negative controlprobes for *Saccharomyces cerevisiae* genes (Act1, Mfa2, Ste3, Ras1) and a 50-mer phage lambda probe (DNA packaging, gil215104 : 7202–7600) spot internal control (Cho *et al*., 2002) were added. All probes were designed using Array Designer 2 (Premier Biosoft International, Palo Alto, CA) using a T_m of 80 ± 5°C, a hairpin ΔG of −6 kcal/mol, a self-dimer ΔG of −12 kcal/mol and a maximum run length of 4. Probes were redesigned if a BLAST match >16 consecutive nucleotides occurred(Kane *et al*., 2000) with entries in the non-redundant database (NCBI)or the *Burkholderia* sp. LB400 genome draft (http://www.jgi-psf.org/draft_microbes/burfu). For hybridization to the spot internal control,a 5′-Cy3 modified complementary lambda probe was designed.

Substrates and array-printing

To select an optimal substrate for spotting the oligonucleotide probes, six types of substrates were used: Superaldehyde (Telechem), poly L-Lysine and epoxysilane (Cel Associates), aminosilane-coated CMT-GAPS II substrates (Corning), 3D-Link activated substrates (Motorola Life Sciences) and thiol-coated EZ-Rays (Apogent Discoversies).

3× SSC (Gibco Ultrapure Reagent) with 0.50 M Betaine (Sigma) (Diehl *et al*., 2001) was used as printing buffer for Superaldehyde, poly L-Lysine, CMT-GAPS II and epoxysilane substrates. Manufacturer's recommended printing buffers were used for 3D-Link and EZ-Ray substrates. Each probe was mixed with the printing buffer in four different concentrations (10, 20, 40 and 60 μM), resulting in the presence of 16 probes per gene, each with a unique combination (= 'probe category') of the three investigated parameters (length, modification and concentration). The internal control lambda probe was added to each spot at a concentration of 10 μM (for artificial community and sensitivity study microarrays) or 20 μM (for all other experiments), modified or unmodified in correspondence to the LB400/RHA1/yeast probe.

Probes were printed in duplicate on each microarray slide (65% relative humidity, 25°C) with an Omnigrid DNA Spotter (Genemachines) at the Genomics Technology Support Facility (Michigan State University). EZ-Rays were activated before printing by reduction of the disulphide bridges to free thiol groups. 3D Link and EZ-Rays were post-processed as described by the manufacturer's protocols, CMT-GAPS II and Poly L-Lysine substrates (Diehl *et al*., 2001) and epoxysilane substrates (Call *et al*., 2001) according to published protocols.

DNA sample preparation

For microarray fabrication optimization, 10 μl samples of a polymerase chain reaction (PCR) product mixture, containing 20 pg μl^{-1} whole length PCR product of each gene (Table 1), except for Ras1 (negative control), were diluted in 1 μg of mouse DNA (Promega).

For labelling optimization, 19 μl of a genomic DNA (gDNA) mixture, containing 10^{-6} pmol μl^{-1} LB400 gDNA (~9 Mbp) and RHA1 gDNA (~6 Mbp) and 10^{-5} pmol μl^{-1} *ohb*, *fcb* plasmid DNA (~12 kbp),was used to which we added 10 pg Act1, 200 pg Ras1 and 600 pg Ste3, 0 pg Mfa2 (negative control).

Labelling for sensitivity analysis hybridizations used varying amounts of the gDNA mixture to which 200 pg Ste3 was added, diluted in mouse gDNA (Promega). The other yeast genes were negative controls (Table 2). This DNA mixture represented an artificial community DNA sample, where 500 ng gDNA corresponds to approximately 10^8 cells of a bacterium with an average genome size (4.6 Mbp). Calculations were made to determine how much gDNA corresponded to 10^6–10^7 cells or 1–10% of the community. Plasmid DNA was added based on the assumption that 10 plasmid copies were present for each copy of the gDNA.

Sediment microcosm study

A sequential anaerobic then aerobic PCB bioremediation strategy was tested in bench scale microcosms. Sediment samples were obtained from the Red Cedar River (MI) and contaminated with 70 μg g^{-1} sediment of Aroclor 1242. The sediment, classified as sand (91.1% sand, 8.1% silt and clay), 6.7% organic matter, 0.71% total nitrogen, pH 7.2, was passed through a 4-mm mesh sieve and stored at 4°C until used. The sediment was inoculated with a PCB-dechlorinating microbial community eluted from the River Raisin sediment and incubated under anaerobic conditions for one year to

Table 1. Description of plasmids, bacterial strains and probes used.

Plasmids, bacterial strains and probes	Characteristics, origin, reference or sequence	Position	Tm	GC%
Plasmids				
pRHD34	*fcbABC* (Rodrigues et al , 2001)			
pRO41	*ohbABCR* (Tsoi *et al.* , 1999)			
Strains				
Escherichia coli DH5aF'	Promega			
Escherichia coli JM109	Promega			
Rhodococcus sp. RHA1	Rodrigues *et al.* (2001)			
Burkholderia sp. LB400	Bopp (1986)			
Genomic DNA				
Saccharomyces cerevisiae	Promega			
70mer probes				
gi|349602:1439-2818,LB400 *bphA*	CACTTACATGGGCGAAGATCCGGTGGTTATGGTGCGACAGAAAGACAAGAGCATCAAGGTGTTCCTGAAC	225	79.8	50
gi|397882:982-1878,LB400 *bphC*	AAACGCATTCATCACTTCATGCTCGAAGTCGCCTCGCTCGATGACGTCGGCTTTGCATTTGATCGGGTTG	616	81.6	51.4
gi|4406503:3354-4640,LB400 *ohbB*	AATTCGACGACAACATCAATCTGGTGGTGCTTTCGATCTTCCCGAACCTGGTGGTGCATCAACTGGGTAA	848	80.3	48.6
gi|510284:180-1562,RHA1 *bphA1*	GACACCTATCTGGGTGAAGCGAAGTTCTACATGGACCACATGTTGGATCGAACCGAAGCGGGCACCGAAG	523	81.5	54.3
gi|510284:4090-5043,RHA1 *bphC*	CCATCTCTCCGACATCATCGACTGGAAGCTCAACTATGAACTGACGGTCAAACTCCACTTCCTGCACTGC	501	80	51.4
gi|1941926:3378-4218,RHA1 *fcbB*	CCGCGCTACGTTCCTTCCTGCCTGGATGTCTATCGGTATCGCCAATGACGCCAGTTCGAGCTTCTACCTC	406	82.5	57.1
gi|14318450:c54695-53260, Yeast Control ACT1	TTTGGATTCCGGTGATGGTGTTACTCACGTCGTTCCAATTTACGCTGGTTTCTCTCTACCTCACGCCATT	744	79.4	47.1
gi|6323989:352411-352527, Yeast Control MFA2	ATGCAACCGATCACCACTGCTTCCACACAAGCCACTCAGAAGGATAAATCCTCTGAAAAGAAAGACAACT	1	78.3	44.3
gi|14318439:515243-516172, Yeast Control RAS1	ACAACGGGTCTTATGTACTCGATAATTCTTTGACCAATGCTGGCACTGGCTCCAGTTCAAAGTCAGCCGT	577	79.5	47.1
gi|14277709:c114629-113217, Yeast Control STE3	CGACGATTTCCTCACGAGATGGGATGGTAAAGGTTGGTGTGATATTGTCATCAAGTTGCAGGTTGGTGCG	111	79.2	48.6
45mer probes				
gi|349602:1439-2818,LB400 *bphA*	CAGGCGGCGTGTTTGAGCAGGACGATGGCGAGAACTGGGTGGAGA	1139	79.8	62.2
gi|397882:982-1878,LB400 *bphC*	GCCGGACGTGACGGTTCCTGCGTACTTCCTGCACTGCAACGAACG	534	79.4	62.2
gi|4406503:3354-4640,LB400 *ohbB*	GCGGCATTGCTTGCTGGCAACCGAACTCCACACCGACGAGGAAGC	720	80	62.2
gi|510284:180-1562,RHA1 *bphA1*	CGAACCTTCTCGGCAGGTGGTGTCTTCGAGCAGGACGACGGCGAG	1096	79.6	64.4
gi|510284:4090-5043,RHA1 *bphC*	GCCGAGCGCGACAGGCAAGTGAGCAGCAACGCAATCGAGGATGAA	865	79.6	60
gi|1941926:3378-4218,RHA1 *fcbB*	TCGCCGCCGCACCCACACACCTCCAAGGACTCGTCAAGAATAGGA	624	79.2	60
gi|14318450:c54695-53260, Yeast Control ACT1	TTTGCCGGTGACGACGCTCCTCGTGCTGTCTTCCCATCTATCGTC	349	77.4	57.8
gi|6323989:352411-352527, Yeast Control MFA2	ATGCAACCGATCACCACTGCTTCCACACAAGCCACTCAGAAGGAT	1	75.7	51.1
gi|14318439:515243-516172, Yeast Control RAS1	TGAAGACGGGTTACGCCTGGCCAAGCAGTTGAATGCACCCTTTCT	359	77	53.3
gi|14277709:c114629-113217, Yeast Control STE3	GCGGCAATATGGAGTGACGACGATTTCCTCACGAGATGGGATGGT	94	75.4	53.3
50 mer lambda probe				
Lambda Probe	ACTGATTGCCCGTCTCCGCTCGCTGGGTGAACAACTGAACCGTGATGTCA			
Cy3-labeled Lambda Target	TGACATCACGGTTCAGTTGTTCACCCAGCGAGCGGAGACGGGCAATCAGT			

Table 2. Experimental design of the sensitivity study. Amounts used to make up the artificial community samples for labelling and the amount of cells or percentage of the community (when assuming 10^8 cells g^{-1} soil) in 1 g soil.

Cells	1.00E+07	5.00E+06	2.50E+06	1.00E+06	Unit
% Community	10.00	5.00	2.50	1.00	%
Chromosome (1 copy/cell)	1.67E-05	8.35E-06	4.18E-06	1.67E-06	pmol
LB400 (~9 Mbp)	89.30	44.65	22.33	8.93	ng
RHA1 (~6 Mbp)	66.10	33.05	16.53	6.61	ng
Plasmid (10 copies/cell)	1.67E-04	8.35E-05	4.18E-05	1.67E-05	pmol
ohb plasmid (~12 kbp)	0.893	0.447	0.223	0.089	ng
fcb plasmid (~12 kbp)	0.893	0.447	0.223	0.089	ng
Postive control Ste3	0.2	0.2	0.2	0.2	ng
Carrier DNA (mouse gDNA)	342	421	461	484	ng
Total	500	500	500	500	ng

achieve substantial PCB dechlorination before being subject to the aerobic stage used here. Total sediment bacterial counts before inoculation with the aerobic cultures were performed by staining with 5-(4,6-dichlorotriazine-2-yl) aminofluoroscein (DTAF) followed by epifluorescence microscopy. Rifampicin-resistant recombinants RHA1(pRHD34) and LB400(pRO41), were grown on 3 mM (nominal concentration) biphenyl-containing medium. Cells were washed once with K1 medium, resuspended, and diluted in the same medium, and 1 ml was added to 1 g of contaminated sediment to give a density of 10^4 cells g^{-1} of sediment for each recombinant strain (separate microcosms for each of the two strains). Samples were taken periodically, and immediately diluted. Appropriate dilutions were spread on Luria–Bertani agar plates containing rifampicin (50 μg ml^{-1}). Numbers of colony forming unit (CFUs) for both strains were determined after one week of incubation. Community DNA was extracted using soil DNA extraction kits (MoBio) and real-time PCR (RT-PCR) counts were performed using Taqman probes targeting *fcbB* or *ohbB* (Rodrigues *et al*., 2002). Hybridizations performed in this study used 200 ng total DNA from the 5 day and 10 day incubation samples for RHA1(pRHD34) and LB400(pRO41) respectively.

DNA sample labelling

To further improve sensitivity, three labelling methods were evaluated. For direct incorporation (enzymatic incorporation of Cy-labeled nucleotides), the Brown protocol for gDNA labelling (http://cmgm.stanford.edu/pbrown/protocols/4_genomic.html) was used. We purified the resulting labeled DNA using ProbeQuant G-50 purification columns (Amersham), according to the manufacturer's instructions.

For amino-allyl labelling (enzymatic incorporation of amino-allyl modified nucleotides, followed by chemical coupling of reactive Cy-dyes) we used a combination of the ARES amino-allyl labelling kit protocol (http://www.probes.com/handbook) and the Brown protocol mentioned above. We used a 1 : 1 ratio between 2-amino-allyl-dUTP (2 mM, Molecular Probes) and dTTP. Microspin G-50 purification columns (Amersham) were used for incorporation reaction cleanup and Qiaquick PCR purification columns (Qiagen) were used for the coupling reaction cleanup, according to the manufacturer's protocol.

TSA labelling (enzymatic incorporation of biotin (or fluorescein)-labelled nucleotides, followed by post-hybridization coupling with a streptavidin (or anti-fluorescein)-horse radish peroxidase complex which enzymatically deposits tyramidecy (dye) used the protocol for direct incorporation with biotin-modified dCTP (1 mM, Perkin Elmer). Purification was performed using ProbeQuant G-50 purification columns (Amersham), except the TSA labelling reactions for the environmental community DNA study that used QIAquick PCR purification columns, after addition of 5 μl 3M NaOAc (pH 5.2–5.5) and the use of 1:10 diluted elution buffer.

Hybridization

CMT-GAPS II and poly L-lysine substrates were washed at 50°C for 15 min in a prehybridization solution (3.5× SSC, 0.1% SDS, 10 mg ml^{-1} BSA), while epoxysilane substrates were prehybridized (5× Denhardt's reagent, 10× SSC) for 30 min at 25°C. Pre-hybridization was concluded with a wash in ddH2O and isopropanol and spinning dry for 2 min (<3000 *g*).

For the microarray fabrication optimization, the hybridization mixture contained 15 pg of Cy3-labelled lambda oligonucleotide and 1/6 of the same Cy5 labelled PCR product mixture, avoiding influence of labelling efficiency on the hybridization. The hybridization buffer consisted of 0.32% SDS (Gibco), 3.36× SSC (Gibco) and 0.8 mg ml^{-1} yeast tRNA (Sigma). For all other hybridizations, 10 pg Cy3-lambda was added to the labelled target in combination with 20 μl *in-situ* hybridization buffer (Agilent). In all cases, the hybridization mixture was denatured for 2 min at 95°C and then chilled on ice. Before applying the mixture (total volume of 40 μl) under the Lifterslip (Erie Scientific), the mixture was preheated at 45°C. Hybridizations were carried out in hybridization cassettes (Telechem), humidified with 30 μl 3× SSC, for 16 h at 65°C.

Post hybridization

EZ-Rays were washed as recommended by the manufacturer. After removing the Lifterslip in 1× SSC, 0.2% SDS at 65°C, all other substrates were washed 5 min in 1× SSC, 0.2% SDS at 65°C, 5 min in 0.1× SSC, 0.2% SDS at 65°C and 1 min in 0.1× SSC at room temperature.

The TSA post-hybridization signal amplification steps used materials provided as part of the Micromax TSA labelling system (Perkin Elmer). Immediately after the washing steps, without spinning the slides dry, the microarray was incubated with 400 μl of TNB-G buffer [mixture of 4.5 ml TN blocking buffer (0.5 g blocking reagent in 100 ml 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.22 μm filtered) and 0.5 ml goat serum (Sigma)] for 10 min to prevent non-specific binding. The slide was washed in TNT buffer [0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20 (Sigma), 0.22 μm filtered] for 1 minute with agitation after which 200 μl streptavidin-HRP conjugate solution [1:100 dilution of streptavidin-HRP conjugate in TNB-G buffer] was applied on the slide and incubated for 10 min. Three rinses for 1 minute with 30 ml TNT buffer were carried out, followed by incubating the slide with 250 μl Cy5 tyramide solution [1:500 Cy5 tyramide (in DMSO) in 1× amplification diluent] for 10 min. Then, the slide was rinsed three times for 5 min in 30 ml of TNT buffer with agitation. After rinsing 1 minute in 30 ml of 0.06× SSC the slide was spun dry (<3000 *g*).

Scanning and data analysis

The slides were scanned with an Axon Genepix 4000a laser scanner with photomultiplier tube (PMT) voltages for microarray fabrication optimization at 600 V for Cy5 (635 nm laser) and 400 V for Cy3 (532 nm laser), for labelling comparisons 600 V (Cy5) and 500 V (Cy3), except for TSA where the Cy5 PMT was lowered to 450. The sensitivity study used a PMT of 480 (Cy5) and 500 (Cy3), while the environmental community DNA study used 600 (Cy5). In all cases, Cy3 signals (lambda internal control), were used to verify the quality of printing and hybridization.

For the microarray fabrication optimization and labelling comparison, data analysis was performed using Excel (Microsoft) and Statview (SAS Company). Each probe spot on the microarray consists of multiple pixels. All data with a [% pixels > background (B) 532 channel + 2 standard deviations (SD)] value smaller than 85% were discarded, as this indicated lower quality of the spot due to failed printing or artifacts resulting from hybridization. The filtered data was ordered in 16 groups or probe categories (per unique combination of microarray fabrication parameters) and Cy5 and Cy3 signals, corrected with the local background, were imported in Statview. Descriptive statistics were calculated and the data were further summarized in Excel.

Data from the sensitivity analysis experiments was eliminated when neither [% pixels > B532 + 2 SD] or [% pixels > B635 + 2 SD] was higher than 70%. To compare results between the different hybridizations of the sensitivity study, the signal of each of the bacterial probes was normalized by division with the nearest Ste3 control signal (local normalization), modified or unmodified and with a spotting concentration, corresponding to the bacterial probe's character. We subtracted this relative signal with the relative signals of the negative controls to correct for non-specific hybridization. Data of the replications on the same slide (duplicate array) and from the replicate hybridization (experimental replicate) were combined and a mean and standard error was calculated for the optimal probe classes.

For the environmental community DNA study, a mean and a standard error of the absolute signals from the duplicate array and the experimental replicate was calculated.

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