Biodegradation (2008) 19:883–895
DOI 10.1007/s10532-008-9190-6

ORIGINAL PAPER

Evaluating the biodegradation of aromatic hydrocarbons by monitoring of several functional genes

Reetta Piskonen · Mari Nyyssönen · Merja Itävaara

Received: 5 July 2007 / Accepted: 1 April 2008 / Published online: 20 April 2008

Abstract Various microbial activities determine the effectiveness of bioremediation processes. In this work, we evaluated the feasibility of gene array hybridization for monitoring the efficiency of biodegradation processes. Biodegradation of ^{14}C-labelled naphthalene and toluene by the aromatic hydrocarbon-degrading *Pseudomonas putida* F1, *P. putida* mt-2 and *P. putida* G7 was followed in mixed liquid culture microcosm by a preliminary, nylon membrane-based gene array. In the beginning of the study, toluene was degraded rapidly and increased amount of toluene degradation genes was detected by the preliminary gene array developed for the study. After toluene was degraded, naphthalene mineralization started and the amount of naphthalene degradation genes increased as biodegradation proceeded. The amount of toluene degradation genes decreased towards the end of the study. The hybridization signal intensities determined by preliminary gene array were in good agreement with mineralization of naphthalene and toluene and with the amount of naphthalene dioxygenase and toluene dioxygenase genes quantified by dot blot hybridization. The clear correlation between the results obtained by the preliminary array and the biodegradation process suggests that gene array methods can be considered as a promising tool for monitoring the efficiency of biodegradation processes.

Keywords Aromatic hydrocarbons · Biodegradation · Functional gene · Monitoring · Process

R. Piskonen (✉) · M. Nyyssönen · M. Itävaara
VTT Technical Research Centre of Finland, Tietotie 2, P. O. Box 1000, 02044 VTT, Espoo, Finland
e-mail: Reetta.Piskonen@wspgroup.fi

M. Nyyssönen
e-mail: Mari.Nyyssonen@vtt.fi

M. Itävaara
e-mail: Merja.Itavaara@vtt.fi

Present Address:
R. Piskonen
WSP Environmental Oy, Heikkiläntie 7, 00210 Helsinki, Finland

Introduction

Microorganisms possess a wide capacity to degrade several types of hydrocarbons, and this can be exploited in the remediation of contaminated environments. Bioremediation has been successfully applied as a decontamination method, and its use may further increase when applied simultaneously with other remediation techniques especially in the case of in situ applications. During bioremediation, the microbial populations responsible for contaminant degradation are influenced by physical, chemical and biological characteristics of the environment. As a result, microbial communities are subjected to

significant changes during the course of biodegradation. Understanding this succession can help us to design more efficient remediation processes for contaminated environments.

Several microbiological methods, such as the enumeration of microorganisms by cultivation on agars plates, determination of the ATP concentrations or general enzyme activities, have been applied for monitoring remediation processes (Piskonen et al. 2002). Even though these methods can be used to monitor the overall microbial activity in the environment, they do not directly describe the enzymatic degradation of specific contaminants.

Microbes use various enzymes for biodegradation of different hydrocarbons. For instance, during the aerobic naphthalene degradation, the aromatic ring is initially activated by naphthalene dioxygenase enzyme. The resulting dihydroxylated intermediate is then converted to catechol that is further oxidized by a catechol 2,3-dioxygenase (Yen and Serdar 1988). The initial oxidation of toluene, on the other hand, is catalyzed by dioxygenase or monooxygenase enzyme (Burlage et al. 1989; Whited and Gibson 1991). Quantifying the genes that are involved in these biodegradation processes with molecular biological methods, such as PCR amplification and hybridization analysis, allow us to monitor biodegradation at the DNA level: contaminant-degrading microorganisms typically become enriched during the course of active biodegradation, and this succession can be studied by analysing the increase in the amount of genes required for the production of the degradative enzymes (Baelum et al. 2006; Piskonen et al. 2005).

However, contamination is often caused by a mixture of hydrocarbons. In order to achieve the goals of bioremediation, several different biodegradative pathways have to function simultaneously. In applications such as monitored natural attenuation, parallel analysis of a number of biodegradative genes is of special interest since, in order to better understand the biodegradation of contaminant mixtures, several degradation pathways need to be monitored concurrently. Determining several different catabolic activities with PCR-based methods is time consuming. New monitoring tools are therefore urgently needed for more comprehensive monitoring of bioremediation processes.

Gene arrays for simultaneously determining the presence of thousands of genes are potential tools for studying the diversity of microbial communities in a range of environments (Brodie et al. 2006; Franke-Whittle et al. 2005; Loy et al. 2005; Wilson et al. 2002). They also hold considerable promise as efficient tools for monitoring microbial succession and functions during bioremediation processes (Brodie et al. 2006; Dennis et al. 2003). Rhee et al. (2004) reported the application of a functional gene array for profiling microbial communities in PAH-contaminated soil after naphthalene enrichment. According to Koizumi et al. (2002), microarray analysis of the consortium degrading toluene and ethylbenzene under sulphate-reducing conditions was consistent with the results given by DGGE and membrane-based hybridization analyses. However, up until now, gene array applications have mainly focused on the characterization of differences in microbial community composition in various environments (Taroncher-Oldenburg et al. 2003; Peplies et al. 2006) or the end-point analysis of contaminant degradation (Denef et al. 2003; Rhee et al. 2004).

So far, glass slide based microarrays have been the main array format in environmental applications because they offer more rapid detection and lower background levels than membrane-based macroarray applications (Shalon et al. 1996). However, due to the low probe-binding capacity of the glass surface, only dominant populations can currently be detected using microarray hybridization (Bodrossy et al. 2003; Denef et al. 2003; Rhee et al. 2004). The inherent variation resulting from different levels of array analysis also limits the comparison of gene copy numbers between samples (Dennis et al. 2003; Gentry et al. 2006). Membrane-based macroarrays, in contrast, may be more suitable for analysing highly diverse microbial communities in the environment. Because the porous array surface possesses a better probe-binding capacity, thereby increasing the amount of matrix-bound probe up to 10^5-fold in comparison to glass-based microarrays, they potentially provide better sensitivity than microarrays (Cho and Tiedje 2002). The lower cost and higher hybridization reproducibility also support their use in biodegradation process monitoring (Steward et al. 2004). Recently, Lievens et al. (2005) described the development of a 18S rRNA gene based DNA macroarray for the quantification of phytopathogenic fungi for plant disease management. Commercial macroarrays have been used for example to profile

genes that are related to osmotic stress in *Escherichia coli* (Weber and Jung 2002). A DNA macroarray for studying nitrogenase gene diversity in water samples has also been reported (Steward et al. 2004).

In order to demonstrate the potential of array analysis in monitoring biodegradation, a nylon membrane-based hybridization assay, hereafter referred to as the preliminary gene array, was developed for analysis of naphthalene and toluene biodegradation at the DNA level. In this preliminary array, several genes from the same pathway were included in order to increase the reliability of the method and to ascertain the presence of whole degradation pathways for the complete biodegradation of the compounds. The suitability of the preliminary gene array for monitoring changes in microbial populations during the biodegradation process was evaluated at laboratory conditions by comparing the changes in the amount of catabolic genes to the mineralization of naphthalene and toluene. The results were also validated by analysing the amount of biodegradative genes by the dot blot hybridization method, as well as by comparing the relative hybridization signals to the microbial community changes observed by denaturing gradient gel electrophoresis (DGGE).

Materials and methods

Bacterial strains

Naphthalene-degrading *Pseudomonas putida* G7 (DSM 4476) and toluene-degrading *P. putida* F1 (DSM 6899) and *P. putida* mt-2 (DSM 3931) were cultivated in Mineral Medium (DSMZ medium 547) at 28°C with 200 mg l^{-1} of naphthalene (Sigma-Aldrich) or toluene (Riedel-de-Haën) as the sole source of carbon. *Bacillus subtilis* (DSM 10), *Escherichia coli* (DSM 1576) and *Pseudomonas putida* (DSM 291) were grown according to the supplier's recommendations. All strains were acquired from the DSMZ culture collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen). *E. coli* strains carrying recombinant plasmids pSSO1genblue, pSSO2genBlue and pCMV5-Mint1 (Jantti et al. 2002) obtained from VTT Technical Research Centre of Finland were grown in Luria-Bertani medium containing 0.1 mg ml^{-1} Ampicillin at 37°C.

Mixed liquid culture microcosm

The *P. putida* strains F1, G7 and mt-2 were grown to the late logarithmic growth phase and the cultures were diluted to 0.08 of A_{600} and mixed in the ratio of 1:1:1. About 5.4 ml of Mineral Medium (DSMZ medium 457), 300 µl of the cell dilution, 50 mg l^{-1} toluene and 50 mg l^{-1} naphthalene were placed in a 30 ml glass vial (Applied Sensor, Sweden) containing a 4 ml vial (Chromacol, UK) with 0.5 ml 0.5 M of NaOH to trap evolved CO_2. The samples were subjected to three treatments: (1) Mineralization of toluene was studied in samples spiked with 7×10^4 dpm [ring-U-^{14}C] toluene (2.8 mCi $mmol^{-1}$) (Sigma, Germany), (2) Mineralization of naphthalene was studied in samples spiked with 180,000 dpm [1-^{14}C]naphthalene (2.3 mCi $mmol^{-1}$) (Sigma, Germany), (3) samples for DNA analysis were not spiked with ^{14}C-labeled substrate. The abiotic control samples were treated with 0.5 ml of 4 N H_2SO_4. The vials were sealed with PTFE/Butyl septa and screw caps (Applied Sensor, Sweden). All the samples were incubated with continuous rotation at 28°C in the dark.

Evolution of $^{14}CO_2$ was measured during a period of 6 days. Four replicate flasks were sacrificed each sampling time to study the mineralization of naphthalene or toluene. The flasks with naphthalene were sampled every 3 h for the first 24 h, and then after 48 and 144 h. The flasks with toluene were sampled after 0, 1, 2, 3, 4, 4.5, 5, 6, 9, 12, 24, 48 and 144 h. In addition, three abiotic control flasks were analyzed every sampling time. 0.5 ml of H_2SO_4 was injected into each flask and the samples were equilibrated on a rotation shaker for one hour at room temperature. The 0.5 ml NaOH trap was mixed with 3 ml of Wallac Optiphase HiSafe 3 Scintillation solution (Wallac, Finland) and the radioactivity was analyzed with a Wallac 1410 scintillation counter (PerkinElmer, Boston, MA, USA). The percentage of mineralization was calculated by dividing the radioactivity in the NaOH trap with the initial amount of radioactive label added to the microcosm.

For DNA hybridization analysis, six samples that had not been spiked with radioactively labelled substrate were taken at each sampling time (0, 2, 3, 4.5, 6, 9, 12, 15, 24 and 144 h). Two samples were pooled and the cells were spun down at 4,000 rpm for 4 min on an Eppendorf 5810R centrifuge, equipped with an A-4-62 rotor (Eppendorf, Hamburg, Germany).

Gas chromatography

At the end of the microcosm study the concentration of toluene and naphthalene was determined by gas chromatography using the static headspace technique. A Tekman 7000 headspace autosampler (Tekmar Dohrmann™, USA) and HP 5890 Series II gas chromatograph (Hewlett Packard, USA) equipped with a flame ionization detector (FID) were used. The samples were equilibrated at 80°C for 30 min. The temperature of the injector was 150°C. Compounds were separated on the HP-5 column (50 m, 0.2 i.d., film thickness 0.33 μm, supplied by Hewlett Packard, USA) with helium as a carrier gas. The GC-oven temperature began at 60°C, was held there for 2 min, then increased at 5°C min^{-1} to 100°C, and then increased at 20°C min^{-1} to 250°C and held there for 5.5 min. The temperature of the detector was 250°C. The pressure was 200 kPa.

DNA preparation

The plasmid DNA was isolated by the alkaline lysis method (Sambrook and Russell 2001), and purified with GeneClean Spin kit (Qbiogene, USA). Total DNA isolation was performed using the bead-beating extraction method as previously described (Stephen et al. 1999). DNA concentration was determined on an Ultrospec 2100 pro spectrophotometer (Biochrom, UK).

Preliminary gene array

The gene probes attached on the preliminary gene array were prepared by PCR amplification using PCR primers previously described in literature: the *nah*A (866 bp) and *nah*E (620 bp) genes involved in the biodegradative pathway for naphthalene were PCR amplified from *P. putida* G7 as previously reported in Ferrero et al. (2002). The *nah*H (962 bp) gene for catechol 2,3-dioxygenase was amplified in a reaction containing 1 × Dynazyme™ II buffer (10 mM Tris–HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl and 1% Triton-X-100), 0.2 mM of each deoxynucleoside triphosphate, 0.1 μM each primer, 1 U Dynazyme™ II DNA polymerase (Finnzymes, Espoo, Finland) and 0.25 μg of plasmid DNA (Ahn et al. 1999). The *tod*C1 (560 bp) gene coding for toluene dioxygenase in *P. putida* F1 was amplified as described in Whyte et al. (1996). The *xyl*A (2,083 bp) and *xyl*E (250 bp) genes for the biodegradation of toluene were amplified from *P. putida* mt-2 using the PCR primers described in Cavalca et al. (2000). Amplification of the *xyl*A gene was performed in a reaction mixture containing 1 × Dynazyme™ II buffer, 0.2 mM each deoxynucleoside triphosphate, 3 mM $MgCl_2$, 7.5% dimethyl sulphoxide, 0.1 μM each primer, 1 U Dynazyme™ II DNA polymerase and 20 ng of plasmid DNA. The PCR program included 30 cycles of 1 min at 94°C, 1 min at 61°C, 2 min at 72°C and a final extension of 10 min at 72°C. Amplification of the *xyl*E gene was carried out as previously described by Cavalca et al. (2000) but with primer concentrations of 2 μM and 50 ng of plasmid DNA. The yeast genes *sso*1 (1,843 bp) and *sso*2 (2,314 bp) were amplified according to Jantti et al. (2002). Amplification of *mint*1 (525 bp) gene was carried out in a reaction mixture composed of 1 × Dynazyme™ II buffer, 0.2 mM of each deoxynucleoside triphosphate, 0.1 μM forward primer 5′-GCATTTCTAGAATGGGACCCT-3′, 0.1 μM reverse primer 5′-GCATTCTCGAGTTAGTTAATCCCGTTGGCCCTGAG-3′, 1 U Dynazyme™ II DNA polymerase and 1 μl 10^{-1} dilution of DNA extract. The PCR program was as follows: 30 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C followed by a final extension of 5 min at 72°C. Following amplification the PCR products were visualized on a 1.2% agarose gel. The properties of the gene probes are described in Tables 1 and 2.

The preliminary gene array was prepared by attaching the PCR-amplified gene probes onto a positively charged Hybond-N+ nylon membrane (Amersham biosciences, UK). About 0.5 μg of catabolic gene probe (*nah*A, *nah*E, *nah*H, *xyl*A, *xyl*E and *tod*C1), negative control probe (*sso*1, *sso*2 and *mint*1) or Lambda-DNA (New England Bio-Labs, UK) was diluted in water, and denatured with 0.45 M NaOH for 10 min at room temperature. The probes were then transferred to the nylon membrane via Bio-Dot Microfiltration Apparatus (Bio-Rad, Hercules, CA, USA). The wells were washed once with 2 × SSC, and the DNA was fixed to the membrane by UV cross-linking (Stratagene, La Jolla, CA, USA). Three replicate spots of each gene probe were blotted onto the membrane to produce a subarray. Each membrane contained two subarrays.

Sample DNA (100 ng) was ^{32}P-labeled using the HexaLabel Plus DNA labelling kit (Fermentas,

Table 1 Gene probes attached on the preliminary gene array

Probe	GenBank	Enzyme	Organism	Substrate/function	Reference
*tod*C1	J04996	Toluene dioxygenase	*Pseudomonas putida* F1	Benzene, toluene, TCE	Zylstra et al. (1989)
*nah*A	M83949	Naphthalene dioxygenase	*Pseudomonas putida* G7	Naphthalene	Simon et al. (1993)
*nah*E	U09057	1,2-Dihydroxybenzylpyruvate aldolase	*Pseudomonas putida* G7	1-Hydroxybenzyl-pyruvate	Eaton (1994)
*nah*H	M17159	Catechol 2,3-dioxygenase	*Pseudomonas putida* G7	Catechol	Ghosal et al. (1987)
*xyl*A	M37480	Xylene monooxygenase	*Pseudomonas putida* mt-2	Toluene, xylenes	Suzuki et al. (1991)
*xyl*E	V01161	Catechol 2,3-dioxygenase	*Pseudomonas putida* mt-2	Catechol	Zukowski et al. (1983)
*mint*1	AF029105	munc18-interacting protein	*Homo sapiens*	Negative control	Aalto et al. (1993)
*sso*1	X67729	Syntaxin 1 homolog	*Sacharomyces cerevisiaie*	Negative control	Aalto et al. (1993)
*sso*2	X67730	Syntaxin 1 homolog	*Sacharomyces cerevisiaie*	Negative control	Okamoto and Sudhof (1997)
Lambda DNA			Bacterio phage Lambda	Positive control	

Table 2 Properties of the gene probes

Probe	Length (bp)	T_m (°C)[a]	GC%
*tod*C1	560	97.0	52
*nah*A	866	98.5	55
*nah*E	620	98.7	56
*nah*H	962	98.1	54
*xyl*A	2,083	96.8	50
*xyl*E	250	98.4	59
*mint*1	525	97.3	53
*sso*1	1,843	92.6	40
*sso*2	2,314	92.3	39

[a] The melting temperature of gene probes was calculated according to Meinkoth and Wahl (1984)

Germany) and 60 μCi [α-^{32}P]dCTP. Prior to labelling, 75 pg of Lambda-DNA (New England Bio-Labs) was included in each labelling reaction to serve as the positive control. Labelled sample DNA was purified by using a NICK column (Amersham Biosciences, UK), and labelling efficiency was determined with a Wallac 1410 liquid scintillation counter (PerkinElmer, Boston, MA, USA).

Hybridization method was optimized by modifying the method developed by Sanseverino et al. (1993). The membranes were prehybridized in 15 ml of hybridization buffer (0.5 M $NaH_2PO_4 \times H_2O$, 1 mM EDTA, 7% SDS, pH 7.2) at 68°C for 90 min in 130 ml roller bottles under continuous rotation in a hybridization oven (Hybaid, Asford, UK). After prehybridization, the entire labelled sample DNA was added to 15 ml of fresh hybridization buffer and the arrays were hybridized in this solution at 68°C overnight. The arrays were washed with 2 × SSC containing 0.1% SDS for 30 min at 68°C, followed by 1 × SSC containing 0.1% SDS for 30 min at 68°C, and the high stringency washing solution for 30 min at 68°C. After exposure on the PhosphorImager screen for 3 h, the screens were scanned by a Typhoon 8600 PhosphoImager (Molecular Dynamics, Sunnyvale, CA, USA).

Hybridization signals were quantified using Imagequant software version 5.2 (Molecular dynamics, Sunnyvale, CA, USA). Spots and background areas were identified manually by circling the spots and background areas with uniformly sized ellipses, after which the background-corrected signal intensities were determined using the Background correction option of the software. Because the ellipses were positioned manually, only spots displaying visible hybridization were included in the analysis.

Prior to data normalization, the Lambda-DNA spots were excluded from the analysis due to high

signal intensity in the spots. After that no statistically significant differences ($P > 0.5$) in the global signal intensities were observed between subarrays. Therefore, the signal intensities of the spots on each array were normalized with the global subarray normalization method applied separately to the two subarrays. Sum of spot intensities on a subarray was determined and mean intensity was calculated by dividing the sum by the number of spots used for the determination. The signal intensity of each spot was divided by the mean intensity within the corresponding subarray to give a proportional signal intensity for the spot. Finally, mean values of six replicate spots on an array were calculated. These mean values were then compared between samples in order to quantify relative differences between the arrays.

Dot blot hybridization

The amount of naphthalene dioxygenase (*nah*A) and toluene dioxygenase (*tod*C1) genes were quantified with the dot blot hybridization analysis described in Piskonen et al. (2005). Total DNA isolated from naphthalene-degrading *P. putida* G7 and toluene-degrading *P. putida* F1 served as the positive control and total DNA isolated from *B. subtilis*, *E. coli* and *P. putida* as negative controls. A standard curve for the quantification of the target genes was established using three independent dilution series of the PCR-amplified *nah*A or *tod*C1 gene fragment (0.03–10 ng). All the samples were blotted onto Hybond-N nylon membrane (Amersham Biosciences, UK) in three replicates.

^{32}P-labeled probes for quantification of the target genes in the membrane-bound DNA samples were generated by PCR amplification. Gene probes were labelled according to Whyte et al. (1996) in a reaction volume of 100 µl. The *tod*C1 gene probe was amplified with 100 µCi and the *nah*A gene probe with 50 µCi of [α-^{32}P]dCTP (Amersham Biosciences, UK) in the reaction mix. The probes were purified with a NICK column (Amersham Biosciences, UK).

Hybridization was performed according to Sanseverino et al. (1993) following the protocol described in Piskonen et al. (2005) as follows: Membranes were hybridized at 68°C for 18 h in 10 ml of hybridization buffer (0.5 M $NaH_2PO_4 \times H_2O$, 1 mM EDTA, 7% SDS, pH 7.2) containing 10–50 µg of heat-denatured probe in a final activity of 5×10^5–1×10^6 dpm ml^{-1}. Following hybridization, the membrane hybridized with the *nah*A probe was washed with 2 × SSC 0.1% SDS for 30 min (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate), with 1 × SSC 0.1% SDS for 30 min and with a high stringency washing solution (10 mM NaCl, 20 mM Tris Base, 1 mM EDTA, 17 mM SDS, pH 7.5) for 30 min at 68°C, and exposed on the PhosphorImager screen (Molecular dynamics, Sunnyvale, CA, USA) for 3 h. The *tod*C1 membrane was washed with 2 × SSC 0.1% SDS for 30 min and with 1 × SSC 0.1%SDS for 30 min at 68°C and exposed on the PhosphorImager screen for 24 h. Scanning and data analysis were performed as previously described (Piskonen et al. 2005).

PCR-DGGE analysis

A 193 bp DNA fragment of the variable region 3 of the 16S rRNA gene was amplified with the P2 and P3 primers described by Muyzer et al. (1993). PCR was carried out in 1 × Dynazyme^{TM} II buffer, 0.2 mM each deoxyribonucleotide triphosphate, 1% formamide, 0.2 µM each primer, 1 U Dynazyme^{TM} II DNA polymerase and 2 µl DNA extract. The thermal profile was 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C.

The amplification products were separated on a 6% polyacrylamide gel in a 40–60% denaturing gradient (100% denaturant is 40% formamide and 7 M urea) in the Bio-Rad DCode System in 0.5 × TAE (Bio-Rad, Hercules, USA). The running conditions were 60 V at 60°C for 18 h. The gel was stained with SYBR Green I (BioWhitaker Molecular Applications, Denmark) and analyzed with Gel Doc 2000 UV-Transilluminator (Bio-Rad, La Jolla, USA).

Statistical analysis

Statistical analysis was performed with the Student's *t*-test.

Results

Naphthalene and toluene biodegradation in the mixed liquid culture microcosm

The biodegradation of toluene and naphthalene in the mixed liquid culture containing *P. putida* strains F1,

mt-2 and G7 was monitored by determining the amount of ^{14}C-carbon mineralized. Mineralization of toluene started immediately after inoculation of the *Pseudomonas* strains, and the plateau phase indicating the end of active mineralization was reached within 12 h when 60% of the toluene was mineralized (Fig. 1). The mineralization of naphthalene was delayed until toluene mineralization had reached the plateau phase after 12 h. The exponential phase of naphthalene mineralization was reached in 22 h, and 60% had been mineralized after 48 h. At the end of the study the concentrations of naphthalene and toluene were below 1 mg l^{-1}, as determined by gas chromatography, whereas in the abiotic controls the hydrocarbon contents were still at the initial concentration of 50 mg l^{-1}.

Specificity of the preliminary gene array

The specificity of the preliminary gene array was studied by hybridization with total DNA extracted from naphthalene-degrading *P. putida* G7 and toluene-degrading *P. putida* mt-2 strains. These strains carry catechol 2,3-dioxygenase genes sharing 80% nucleotide sequence similarity in the NAH7 and pWW0 plasmids, respectively (Harayama et al. 1987). For both degradation pathways specific hybridization to corresponding gene probes was detected. No significant cross hybridization was observed with any of the genes, and no hybridization to negative control genes occurred.

Biodegradation process monitoring using the preliminary gene array

In order to evaluate the suitability of gene array analysis for monitoring biodegradation processes, DNA samples from four different data points in the course of degradation were analysed on the preliminary gene array. The first three replicate samples were taken 9 h after the start of the experiment when toluene mineralization had reached its maximum and naphthalene mineralization was beginning. DNA samples taken after 15 h describe the exponential phase of naphthalene biodegradation. After 24 h, naphthalene biodegradation had reached the plateau phase. The fourth samples were taken at the end of the study.

The hybridization signals were readily detected on the nylon membranes, as can be seen in Fig. 2a, where the DNA sample taken after 15 h was hybridized on the preliminary gene array. No hybridization signal was observed in the negative control genes *sso*1, *sso*2 and *mint*1 (Fig. 2b).

The relative signal intensity in the gene probes encoding degradation of toluene (*xyl*A = 1.23,

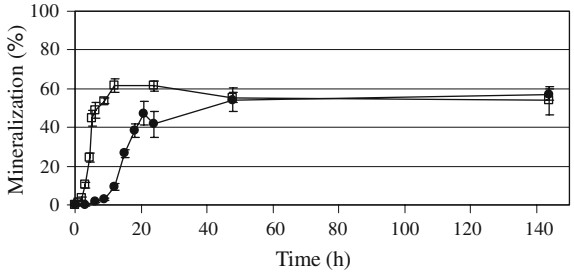

Fig. 1 Mineralization of ^{14}C-labelled toluene (□) and naphthalene (●) during the course of the microcosm study

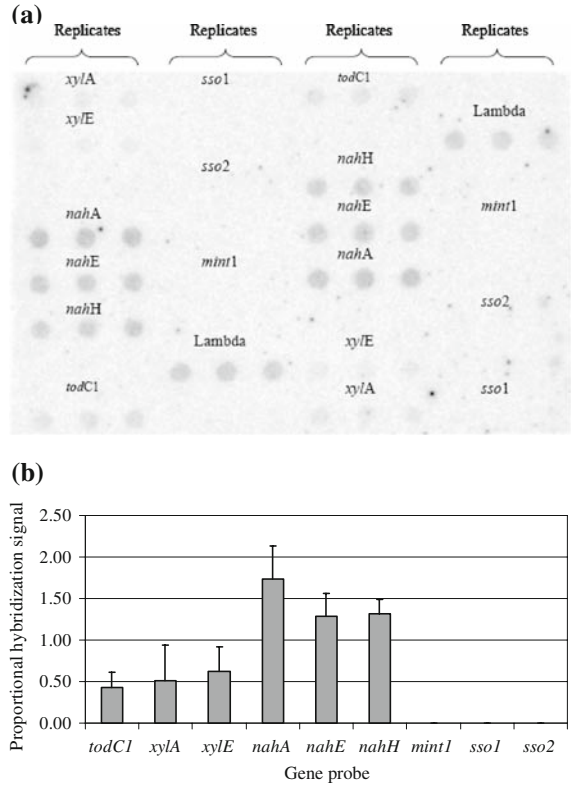

Fig. 2 Hybridization of the DNA sample on the preliminary gene array. The DNA was extracted from the sample withdrawn 15 h after the experiment was started: (**a**) The membrane after hybridization (**b**) The proportional hybridization signals detected on the corresponding membrane

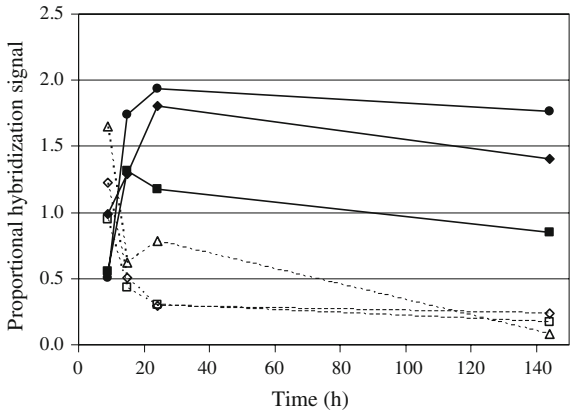

Fig. 3 The mean proportional hybridization signals in catabolic gene probes during the course of the biodegradation process as observed by the preliminary gene array analysis. The changes in *tod*C1 (□), *xyl*A (◇) and *xyl*E (Δ) genes involved in the degradation of toluene are indicated with dashed lines. The changes in naphthalene catabolic genes, *nah*A (●), *nah*E (♦), *nah*H (■), are indicated with a solid line

*xyl*E = 1.65 and *tod*C1 = 0.95) were statistically significantly higher ($0.001 < P < 0.05$) than that of genes from the *nah* pathway (*nah*A = 0.51, *nah*E = 0.99, *nah*H = 0.55) in samples withdrawn after 9 h, except the *nah*E gene (Fig. 3). After naphthalene mineralization started, the proportion of *nah*A, *nah*E, and *nah*H genes increased to 1.94, 1.80, and 1.18, respectively, whereas the proportional hybridization signals in the *tod*C1, *xyl*A, and *xyl*E genes responsible for toluene degradation decreased to 0.30, 0.29, and 0.78, respectively. After 24 h, when naphthalene mineralization had ceased, the signal intensity in the *nah*-probes decreased, but was still significantly higher than in the probes encoding genes for toluene biodegradation. The results obtained by gene array were in good agreement with the mineralization data.

In order to evaluate the sensitivity of the array, relative amounts of *nah*A and *tod*C1 genes determined by the gene array were compared to the amount of target genes detected by dot blot hybridization. According to dot blot analysis DNA samples recovered from the microcosms after 9 h contained 9 pg of *nah*A gene, and detectable hybridization signals were measured in the *nah*A gene probe. In the *tod*C1 gene probe, DNA samples containing 280 pg of target gene produced measurable hybridization signals at the end of the study.

Table 3 Proportional hybridization signal intensities in the functional gene probes attached on the preliminary gene array in the course of the biodegradation study

Gene probe	Time (h)	Time (h)			
		9	15	24	144
*tod*C1		1.23	0.51	0.29	0.24
	9		**	***	***
	15			**	***
	24				*
*xyl*A		1.65	0.62	0.78	0.08
	9		NSD	*	**
	15			**	**
	24				NSD
*xyl*E		1.65	0.62	0.78	0.08
	9		**	*	***
	15			NSD	***
	24				***
*nah*A		0.51	1.74	1.94	1.76
	9		***	***	***
	15			NSD	NSD
	24				**
*nah*E		0.99	1. 29	1.80	1.41
	9		NSD	**	NSD
	15			***	NSD
	24				***
*nah*H		0.55	1.32	1.18	0.85
	9		***	*	NSD
	15			*	***
	24				*

The statistical significance of differences signal intensities in course of time were determined by the Student's *t*-test. The signal intensity in the probe from three replicate DNA extracts was compared to that in the same probe at another time point. The *P*-values are indicated with asterisks (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$). NSD = No statistically significant difference observed

The Student's *t*-test performed on the preliminary gene array data showed that the differences observed in the relative hybridization signal on a certain gene probe during the course of the biodegradation study were statistically significant (Table 3). For example, the relative hybridization signal intensity in the *tod*C1 gene probe after 9 h differed significantly from the relative signal intensities observed after 15 h ($P < 0.005$) and 24 h ($P < 0.0005$). Statistically significant difference was observed also between 15 h and 144 h. Even the signal in the *tod*C1 probe

after 24 h differed from the signal observed after 144 h ($P < 0.05$).

Validation of microbial succession by dot blot hybridization and PCR-DGGE analysis

In order to validate the results obtained by the preliminary gene array, the amount of *tod*C1 and *nah*A genes were determined by dot blot hybridization during the biodegradation process. An exponential increase in the amount of *tod*C1 gene responsible for degradation of toluene was detected by dot blot hybridization when toluene biodegradation started (Fig. 4). The amount of *tod*C1 gene increased from 0.5 to 7.7 ng (μg total DNA)$^{-1}$ during 9 h (Fig. 4a). Thereafter, the growth of *P. putida* F1 was reduced due to substrate depletion, and the amount of *tod*C1 genes decreased. Simultaneously as the amount of the *tod*C1 genes declined, dot blot hybridization showed that the numbers of the *nah*A genes increased until naphthalene biodegradation was complete (Figs. 1 and 4b). After reaching 1.4 ng (μg total DNA)$^{-1}$, the numbers of *nah*A genes seemed to remain at this maximum level and the drop in the amount of *tod*C1 also ceased. The amount of *tod*C1 and *nah*A genes was in good agreement with the exponential mineralization rates observed for toluene and naphthalene. There also was a strong indication that the correlation between the amount of the genes in the dot blot analysis and in the gene-array was significant (Fig. 4), since for *tod*C1 $r^2 = 0.93$ and for *nah*A $r^2 = 0.93$.

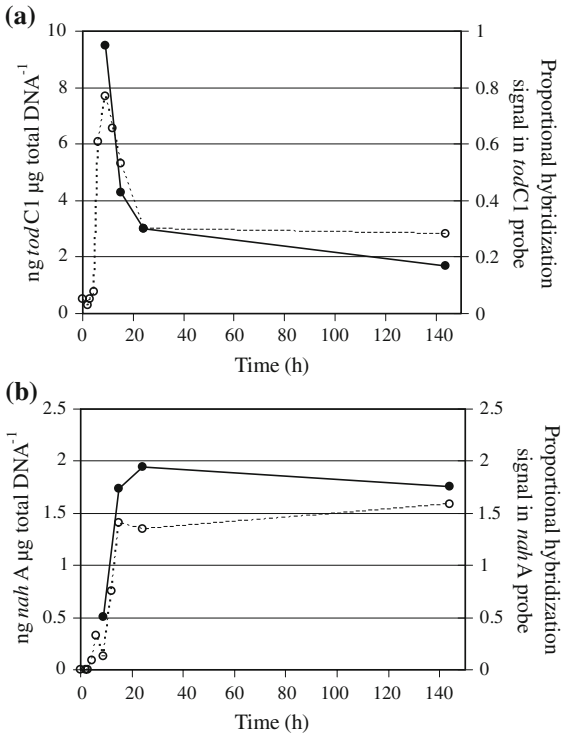

Fig. 4 The mean amount of (**a**) *tod*C1 and (**b**) *nah*A genes during the course of the microcosm study. The concentration of target gene was determined by dot blot hybridization (○) and proportional hybridization signal by the preliminary gene array (●)

Microbial succession observed by PCR-DGGE was in good agreement with the mineralization and dot blot hybridization data. Initially, all the strains were observed in the microbial community profile (Fig. 5). The 16S rRNA gene fragment of *P. putida* F1 carrying the *tod* pathway for toluene degradation in the chromosomal DNA was most prevalent band on DGGE after 6 h. As toluene mineralization proceeded, the 16S rRNA gene fragment of *P. putida* mt-2 could not be detected by DGGE method. After 15 h, a 16S rRNA gene fragment corresponding to *P. putida* G7 appeared on the DGGE gel indicating a shift in the microbial community structure between 9 and 15 h (Fig. 5). At this stage, toluene mineralization was completed and naphthalene mineralization started.

Discussion

Effective monitoring of environmental microbial processes requires the ability to differentiate between target sequences with a high sensitivity. Although carefully designed oligonucleotide microarrays enable differentiation of target sequences containing one mismatch (Urakawa et al. 2003), the detection limit of oligonucleotide probes has been found to vary between 1% and 5% of the total microbial community, thereby enabling the detection of dominant populations only (Denef et al. 2003; Franke-Whittle et al. 2005; Rhee et al. 2004). In this study, we used PCR amplified probes to establish the preliminary gene array because longer probes offer better sensitivity than short oligonucleotide probes (Letowski et al. 2004). As a result, a clear hybridization signal was detected in the *nah*A probe with 9 pg of target gene, indicating that the detection limit,

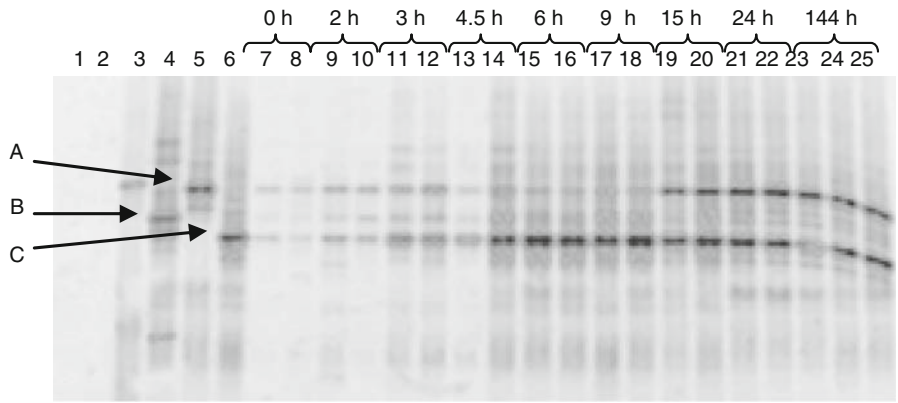

Fig. 5 The structure of the microbial community during the course of the microcosm study as determined by the DGGE analysis. Arrows indicate positive control samples PCR amplified from (A) *P. putida* G7, (B) *P. putida* mt-2 and (C) *P. putida* F1 total DNA

even though not determined in our study, seems to be at the picogram level.

Because the specificity of hybridization depends on the hybridization characteristics of the probe (Letowski et al. 2004), the probes attached on the preliminary gene array were selected to have as similar melting temperatures as possible. The analysis performed with the pure cultures showed that *nah*H and *xyl*E genes sharing 80% nucleotide sequence similarity could be differentiated under the highly stringent conditions (Harayama et al. 1987). This is in good accordance with the results of Wu et al. (2001), who demonstrated that 80–85% similar gene fragments can be differentiated under highly stringent hybridization conditions. A small variation in the GC content, and thus in the melting temperature, seems to be acceptable since, according to Wu et al. (2001), the probes whose GC contents differed from each other by 8% did not produce significantly different hybridization signals. Our results supported this observation, since a difference of 9% in the GC content corresponding to a 1.6°C difference in the melting temperature between the *xyl*A and *xyl*E gene probes did not seem to have any significant effect on the hybridization. As a result of similar hybridization characteristics, the proportional changes in the genes from the same pathway were in good agreement with each other demonstrating that inclusion of several gene probes from the same pathway on the array increased the reliability of the method and enabled the targeting of the whole degradation pathways.

In order to evaluate the suitability of gene array methods for monitoring biodegradation processes, the preliminary gene array was applied for monitoring of the biodegradation of toluene and naphthalene in liquid medium by a microbial consortium containing three biodegradative *P. putida* strains. Gene array analysis was in good agreement with dot blot hybridization as well as with the mineralization process, where naphthalene mineralization was observed after toluene biodegradation had ceased. All the methods indicated that there was a significant shift in microbial community as naphthalene biodegradation started. Similar phenomenon was observed by Bouchez et al. (1995) on biodegradation of some PAH mixtures with microbial consortium. According to them, toxicity of naphthalene inhibited growth of strains not isolated on naphthalene and after depletion of this toxic substrate the biodegradation of other compounds was initiated.

However, slightly contradictory results were observed by PCR-DGGE analysis. The PCR-DGGE analysis indicated prevalence of *P. putida* F1 carrying the *tod* pathway during toluene degradation whereas according to the gene array analysis the catabolic genes from both toluene degradation pathways were present in relatively similar amounts. Also, both preliminary array and dot blot analyses showed that the proportional amount of *P. putida* strains F1 and mt-2 in the mixed culture declined as naphthalene degradation proceeded and the proportional amount of *P. putida* G7 increased whereas according to the PCR-DGGE analysis *P. putida* G7 was accompanied by *P. putida* F1 until the end of the study. It is possible that PCR amplification of the ribosomal RNA genes for DGGE analysis may have more effectively amplified the 16S rRNA gene from *P. putida* F1 than that from *P. putida* mt-2. Suzuki and Giovannoni (1996) demonstrated that while

DGGE is good for monitoring microbial community succession during contaminant biodegradation the method cannot be reliably used for quantitative analysis of specific bacterial phylotypes due to the bias in PCR amplification. Detection of *P. putida* F1 at the end of the study may also be caused by the stability of DNA resulting in PCR amplification of bacterial DNA from the dead microbial biomass.

It is also possible that the difference observed between gene array and DGGE analysis is due to the different hybridization efficiencies between the probes from the *tol* pathway and the *tod* pathway. This may be due to different labelling efficiencies of the target nucleic acids (Martin-Magniette et al. 2005), to accessibility differences for the different probe target sites due to secondary structures of the target DNA (Lane et al. 2004), or to steric hindrances on the different nucleic acid hybrids on the array (Shchepinov et al. 1997). Taroncher-Oldenburg et al. (2003) suggested the use of probes with equal length and minimal secondary structure in order to further equalize the signal intensities between different probes. In our study, the length of the probes varied between 250 and 2,214 bp. According to Wu et al. (2001), the signal intensity increases linearly as the size of the probe increases up to about 1 kb. However, the increase in the signal intensity as the size of the probe increases was not seen in our study. For example, the signals obtained from the *xyl*A (2,083 bp) and *xyl*E (250 bp) did not differ from each other significantly, and similar changes in the signal intensities were observed during the course of the study.

Previously, Taniguchi et al. (2001) reported that the data obtained by the gene array were in good agreement with the results of Northern blot analysis. Our results also demonstrated that there was a significant correlation between the preliminary array and dot blot analyses. However, in order to obtain statistically significant results from highly heterogeneous environments, attention should be paid to array-to-array normalization and the number of replicate analyses (Cook and Sayler 2003; Lee et al. 2000). Proper normalization that minimizes the variation between slides due to spotting unevenness or differences in labelling and hybridization efficiencies and variations during image analysis is especially important when studying diverse microbial communities in the environment (Cook and Sayler 2003). On the preliminary gene array there were two separate subarrays, each containing three probes for every gene, and each subarray was normalized separately. This decreased the variation of the results and therefore increased their reliability. Clear correlation between the gene array and dot blot analysis was, however, obtained only after three biological replicate samples were analysed. This indicates that an adequate number of true replicates should always be included when biological processes are analysed. In this study, lambda DNA served as an internal control to the labelling reaction in order to confirm the success of labelling and hybridization.

According to our previous studies, the efficiency of biodegradation process can be evaluated by monitoring the changes in the number of catabolic genes involved in the degradation (Piskonen et al. 2005). On the basis of this preliminary study the simultaneous analysis of several catabolic genes by gene array consisting of long PCR-amplified gene probes is a promising method for monitoring the succession of several different contaminant-degrading bacteria during biodegradation processes. Yet, while the changes in the hybridization signals could be related to mineralization of the hydrocarbons, application of the gene arrays for monitoring of the activity of contaminant-degrading microorganisms during true environmental processes still need to be studied. The targeted bacteria often represent only a small fraction of the heterogeneous environmental microbial community and arrays with high sensitivity are required. The construction of functional gene arrays covering even the most relevant genotypes present in the environment is also a challenge, since functional diversity in the environment is only partially known. In addition, better array-to-array comparison and more reliable quantification of results requires development of a more sophisticated internal control based normalization method. Suitability of gene arrays for monitoring of catabolic activities on RNA-level in environmental samples is also a challenge. For that purpose, new methods for extraction sufficient amounts of high quality mRNA from environmental samples are required. Nevertheless, gene array technology is developing rapidly and these restrictions will almost certainly be overcome. Even analysis in field conditions by portable microarray systems may be applied in monitoring the efficiency biodegradation processes in the future (Bavykin et al. 2001).

Acknowledgements Funding for the research was provided by the Neste Foundation, the Ekokem Foundation, the Finnish Funding Agency for Technology and Innovation (TEKES) and VTT's Strategic Clean World Tecnology Theme, T2 In situ Project. Phil.Lic. Tiina Rajamäki is acknowledged for developing the gas chromatography method for analysing toluene and naphthalene. Mrs. Anna-Liisa Ruskeepää is thanked for performing the toluene and naphthalene analyses. Dr. Jussi Jäntti is thanked for providing the *E. coli* strains carrying recombinant plasmids, from which the negative control genes were isolated.

References

Aalto MK, Ronne H, Keranen S (1993) Yeast syntaxins Sso1p and Sso2p belong to a family of related membrane proteins that function in vesicular transport. EMBO J 12(11):4095–4104

Ahn Y, Sanseverino J, Sayler GS (1999) Analyses of polycyclic aromatic hydrocarbon-degrading bacteria isolated from contaminated soils. Biodegradation 10(2):149–157

Baelum J, Henriksen T, Hansen HC, Jacobsen CS (2006) Degradation of 4-chloro-2-methylphenoxyacetic acid in top- and subsoil is quantitatively linked to the class III tfdA gene. Appl Environ Microbiol 72(2):1476–1486

Bavykin SG, Akowski JP, Zakhariev VM, Barsky VE, Perov AN, Mirzabekov AD (2001) Portable system for microbial sample preparation and oligonucleotide microarray analysis. Appl Environ Microbiol 67(2):922–928

Bodrossy L, Stralis-Pavese N, Murrell JC, Radajewski S, Weilharter A, Sessitsch A (2003) Development and validation of a diagnostic microbial microarray for methanotrophs. Environ Microbiol 5(7):566–582

Bouchez M, Blanchet D, Vandecasteele JP (1995) Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations: inhibition phenomena and cometabolism. Appl Microbiol Biotechnol 43(1):156–164

Brodie EL, Desantis TZ, Joyner DC et al (2006) Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. Appl Environ Microbiol 72(9):6288–6298

Burlage RS, Hooper SW, Sayler GS (1989) The TOL (pWW0) catabolic plasmid. Appl Environ Microbiol 55(6):1323–1328

Cavalca L, Di Gennaro P, Colombo M et al (2000) Distribution of catabolic pathways in some hydrocarbon-degrading bacteria from a subsurface polluted soil. Res Microbiol 151(10):877–887

Cho JC, Tiedje JM (2002) Quantitative detection of microbial genes by using DNA microarrays. Appl Environ Microbiol 68(3):1425–1430

Cook KL, Sayler GS (2003) Environmental application of array technology: promise, problems and practicalities. Curr Opin Biotechnol 14(3):311–318

Denef VJ, Park J, Rodrigues JL, Tsoi TV, Hashsham SA, Tiedje JM (2003) Validation of a more sensitive method for using spotted oligonucleotide DNA microarrays for functional genomics studies on bacterial communities. Environ Microbiol 5(10):933–943

Dennis P, Edwards EA, Liss SN, Fulthorpe R (2003) Monitoring gene expression in mixed microbial communities by using DNA microarrays. Appl Environ Microbiol 69(2):769–778

Eaton RW (1994) Organization and evolution of naphthalene catabolic pathways: sequence of the DNA encoding 2-hydroxychromene-2-carboxylate isomerase and trans-o-hydroxybenzylidenepyruvate hydratase-aldolase from the NAH7 plasmid. J Bacteriol 176(24):7757–7762

Ferrero M, Llobet-Brossa E, Lalucat J, Garcia-Valdes E, Rossello-Mora R, Bosch R (2002) Coexistence of two distinct copies of naphthalene degradation genes in Pseudomonas strains isolated from the western Mediterranean region. Appl Environ Microbiol 68(2):957–962

Franke-Whittle IH, Klammer SH, Insam H (2005) Design and application of an oligonucleotide microarray for the investigation of compost microbial communities. J Microbiol Methods 62(1):37–56

Gentry TJ, Wickham GS, Schadt CW, He Z, Zhou J (2006) Microarray applications in microbial ecology research. Microb Ecol 52(2):159–175

Ghosal D, You IS, Gunsalus IC (1987) Nucleotide sequence and expression of gene nahH of plasmid NAH7 and homology with gene xylE of TOL pWWO. Gene 55(1):19–28

Harayama S, Rekik M, Wasserfallen A, Bairoch A (1987) Evolutionary relationships between catabolic pathways for aromatics: conservation of gene order and nucleotide sequences of catechol oxidation genes of pWW0 and NAH7 plasmids. Mol Gen Genet 210(2):241–247

Jantti J, Aalto MK, Oyen M, Sundqvist L, Keranen S, Ronne H (2002) Characterization of temperature-sensitive mutations in the yeast syntaxin 1 homologues Sso1p and Sso2p, and evidence of a distinct function for Sso1p in sporulation. J Cell Sci 115(Pt 2):409–420

Koizumi Y, Kelly JJ, Nakagawa T et al (2002) Parallel characterization of anaerobic toluene- and ethylbenzene-degrading microbial consortia by PCR-denaturing gradient gel electrophoresis, RNA-DNA membrane hybridization, and DNA microarray technology. Appl Environ Microbiol 68(7):3215–3225

Lane S, Evermann J, Loge F, Call DR (2004) Amplicon secondary structure prevents target hybridization to oligonucleotide microarrays. Bios Bioelectron 20(4):728–735

Lee ML, Kuo FC, Whitmore GA, Sklar J (2000) Importance of replication in microarray gene expression studies: statistical methods and evidence from repetitive cDNA hybridizations. Proc Natl Acad Sci USA 97(18):9834–9839

Letowski J, Brousseau R, Masson L (2004) Designing better probes: effect of probe size, mismatch position and number on hybridization in DNA oligonucleotide microarrays. J Microbiol Methods 57(2):269–278

Lievens B, Brouwer M, Vanachter AC, Levesque CA, Cammue BP, Thomma BP (2005) Quantitative assessment of phytopathogenic fungi in various substrates using a DNA macroarray. Environ Microbiol 7(11):1698–1710

Loy A, Schulz C, Lucker S et al (2005) 16S rRNA gene-based oligonucleotide microarray for environmental monitoring of the betaproteobacterial order "Rhodocyclales". Appl Environ Microbiol 71(3):1373–1386

Martin-Magniette ML, Aubert J, Cabannes E, Daudin JJ (2005) Evaluation of the gene-specific dye bias in cDNA microarray experiments. Bioinformatics (Oxford, England) 21(9):1995–2000

Meinkoth J, Wahl G (1984) Hybridization of nucleic acids immobilized on solid supports. Anal Biochem 138(2):267–284

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59(3):695–700

Okamoto M, Sudhof TC (1997) Mints, Munc18-interacting proteins in synaptic vesicle exocytosis. J Biol Chem 272(50):31459–31464

Peplies J, Lachmund C, Glockner FO, Manz W (2006) A DNA microarray platform based on direct detection of rRNA for characterization of freshwater sediment-related prokaryotic communities. Appl Environ Microbiol 72(7):4829–4838

Piskonen R, Kapanen A, Mansikka T, Rytkonen J, Itavaara M (2002) Evaluation of bioremediation treatments in a Shoreline-Simulating Microcosm. Biorem J 6(2):143–158

Piskonen R, Nyyssonen M, Rajamaki T, Itavaara M (2005) Monitoring of accelerated naphthalene-biodegradation in a bioaugmented soil slurry. Biodegradation 16(2):127–134

Rhee SK, Liu X, Wu L, Chong SC, Wan X, Zhou J (2004) Detection of genes involved in biodegradation and biotransformation in microbial communities by using 50-mer oligonucleotide microarrays. Appl Environ Microbiol 70(7):4303–4317

Sambrook J, Russell D (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, New York, US

Sanseverino J, Werner C, Fleming J, Applegate B, King JMH, Sayler GS (1993) Molecular diagnostic of polycyclic aromatic hydrocarbon biodegradation in manufactured gas plant soils. Biodegradation 4:303–321

Shalon D, Smith SJ, Brown PO (1996) A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. Genome Res 6(7):639–645

Shchepinov MS, Case-Green SC, Southern EM (1997) Steric factors influencing hybridisation of nucleic acids to oligonucleotide arrays. Nucleic Acids Res 25(6):1155–1161

Simon MJ, Osslund TD, Saunders R et al (1993) Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB 9816-4. Gene 127(1):31–37

Stephen JR, Chang YJ, Gan YD et al (1999) Microbial characterization of a JP-4 fuel-contaminated site using a combined lipid biomarker/polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE)-based approach. Environ Microbiol 1(3):231–241

Steward GF, Jenkins BD, Ward BB, Zehr JP (2004) Development and testing of a DNA macroarray to assess nitrogenase (nifH) gene diversity. Appl Environ Microbiol 70(3):1455–1465

Suzuki MT, Giovannoni SJ (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl Environ Microbiol 62(2):625–630

Suzuki M, Hayakawa T, Shaw JP, Rekik M, Harayama S (1991) Primary structure of xylene monooxygenase: similarities to and differences from the alkane hydroxylation system. J Bacteriol 173(5):1690–1695

Taniguchi M, Miura K, Iwao H, Yamanaka S (2001) Quantitative assessment of DNA microarrays—comparison with Northern blot analyses. Genomics 71(1):34–39

Taroncher-Oldenburg G, Griner EM, Francis CA, Ward BB (2003) Oligonucleotide microarray for the study of functional gene diversity in the nitrogen cycle in the environment. Appl Environ Microbiol 69(2):1159–1171

Urakawa H, El Fantroussi S, Smidt H et al (2003) Optimization of single-base-pair mismatch discrimination in oligonucleotide microarrays. Appl Environ Microbiol 69(5):2848–2856

Weber A, Jung K (2002) Profiling early osmostress-dependent gene expression in Escherichia coli using DNA macroarrays. J Bacteriol 184(19):5502–5507

Whited GM, Gibson DT (1991) Toluene-4-monooxygenase, a three-component enzyme system that catalyzes the oxidation of toluene to p-cresol in Pseudomonas mendocina KR1. J Bacteriol 173(9):3010–3016

Whyte LG, Greer CW, Inniss WE (1996) Assessment of the biodegradation potential of psychrotrophic microorganisms. Can J Microbiol 42(2):99–106

Wilson KH, Wilson WJ, Radosevich JL et al (2002) High-density microarray of small-subunit ribosomal DNA probes. Appl Environ Microbiol 68(5):2535–2541

Wu L, Thompson DK, Li G, Hurt RA, Tiedje JM, Zhou J (2001) Development and evaluation of functional gene arrays for detection of selected genes in the environment. Appl Environ Microbiol 67(12):5780–5790

Yen KM, Serdar CM (1988) Genetics of naphthalene catabolism in pseudomonads. Crit Rev Microbiol 15(3):247–268

Zukowski MM, Gaffney DF, Speck D et al (1983) Chromogenic identification of genetic regulatory signals in *Bacillus subtilis* based on expression of a cloned Pseudomonas gene. Proc Natl Acad Sci USA 80(4):1101–1105

Zylstra GJ, Wackett LP, Gibson DT (1989) Trichloroethylene degradation by *Escherichia coli* containing the cloned *Pseudomonas putida* F1 toluene dioxygenase genes. Appl Environ Microbiol 55(12):3162–3166