MICROBIAL ECOLOGY

Microb Ecol (2003) 46:161–176
DOI: 10.1007/s00248-003-2005-8

Bacterial Community Dynamics during Biostimulation and Bioaugmentation Experiments Aiming at Chlorobenzene Degradation in Groundwater

D.F. Wenderoth, P. Rosenbrock, W.-R. Abraham, D.H. Pieper, M.G. Höfle

Department for Environmental Microbiology, GBF-German Research Centre for Biotechnology, Mascheroder Weg 1, D-38124 Braunschweig, Germany

Received: 21 August 2002; Accepted: 30 January 2003; Online publication: 6 June 2003

ABSTRACT

A set of microcosm experiments was performed to assess different bioremediation strategies, i.e., biostimulation and bioaugmentation, for groundwater contaminated with chlorobenzenes. The biodegradative potential was stimulated either by the supply of electron acceptors (air, (NO_3^-), to increase the activity of the indigenous bacterial community, or by the addition of aerobic chlorobenzene-degrading bacteria (*Pseudomonas putida* GJ31, *Pseudomonas aeruginosa* RHO1, *Pseudomonas putida* F1ΔCC). Experiments were performed with natural groundwater of the aquifer of Bitterfeld, which had been contaminated with 1,2-dichlorobenzene (1,2-DCB), 1,4-dichlorobenzene (1,4-DCB), and chlorobenzene (CB). The microcosms consisted of airtight glass bottles with 800 mL of natural groundwater and were incubated under *in situ* temperature (13°C). Behavior of the introduced strains within the indigenous bacterial community was monitored by fluorescent *in situ* hybridization (FISH) with species-specific oligonucleotides. Dynamics of the indigenous community and the introduced strains within the microcosms were followed by single-strand conformation polymorphism (SSCP) analysis of 16S rDNA amplicons obtained from total DNA of the microbial community. An indigenous biodegradation potential under aerobic as well as anaerobic denitrifying conditions was observed accompanied by fast and specific changes in the natural bacterial community composition. Augmentation with *P. aeruginosa* RHO1 did not enhance bio-degradation. In contrast, both *P. putida* GJ31 as well as *P. putida* F1ΔCC were capable of growing in groundwater, even in the presence of the natural microbial community, and thereby stimulating chlorobenzene depletion. *P. putida* GJ31 disappeared when the xenobiotics were depleted and *P. putida* F1ΔCC persisted even in the absence of CB. Detailed statistical analyses revealed that community dynamics of the groundwater microbiota were highly reproducible but specific to the introduced strain, its inoculum size, and the imposed physicochemical conditions. These findings could contribute to the design of better *in situ* bioremediation strategies for contaminated groundwater.

Correspondence to: D.F. Wenderoth; E-mail: dfw@gbf.de

Introduction

Contamination of aquifers with halogenated organic compounds is a widespread problem arising from the extensive use of pesticides in agriculture or from industrial chemical waste. The contaminations in the aquifer of Bitterfeld, near Leipzig, Germany, derived from the chemical industry in this area and has persisted for several decades. The pollution extends over an area of 25 km^2 and is distributed in about 200 million m^3 of groundwater [47]. The quaternary aquifer, which is contaminated by chlorinated benzenes, extends from a depth of about 5 to 20 m and is confined at a depth of 20 m by a lignite layer that serves as a hydraulic barrier for the aquifer [32] and reacts as an adsorbing surface for the hydrophobic chlorobenzenes. Chlorobenzenes adsorbed to the lignite layer are in equilibrium with the aqueous phase and thus continuously redelivered. Therefore, the classical approach to remediate the groundwater by "pump and treat" is not economically viable.

Bioremedation of contaminated environments like aquifers by microorganisms has become an accepted technology for the restoration of these environments [48]. A conceivable bioremedation strategy is stimulation of the indigenous biodegradation potential by the addition of suitable electron donors/acceptors [29] or nutrients [33]. However, bioremediation by biostimulation requires that a natural potential of the indigenous microbial community exist to degrade the pollutant. Therefore, successful applications have primarily involved readily degradable organic compounds [6, 8] such as natural oil and diesel fuel. It can be assumed that bacteria with the capability to mineralize chlorobenzenes are not ubiquitous or are present in low numbers. Where the appropriate degradation potential is not present, bioaugmentation can accelerate the removal of undesired compounds from contaminated sites or bioreactors by introducing specialized indigenous, allochthonous wild-type or genetically engineered strains [4, 44].

The use of microbial inocula to stimulate degradation in aquifers has been demonstrated in pilot field tests for TCE degradation using methanotrophic bacteria [13] and for CCl_4 degradation by a denitrifying *Pseudomonas* strain [15]. However, the bioaugmentation approach is discussed critically in respect to its efficiency and usefulness. Whereas inocula are usually highly efficient in the removal of the target compounds under laboratory conditions, their performance under natural conditions cannot be predicted. In some cases bioaugmentation has been successful in accelerating the removal of target xenobiotics under environmental conditions [4, 22], whereas in other cases, introduced specialized strains survived poorly and failed to develop their biodegradation activity [37, 45].

In the present study groundwater microcosms were used to assess the effect of different bio-stimulation and bioaugmentation approaches on the community structure and dynamics of indigenous bacterial communities in parallel with the biodegradation processes. Indigenous communities were stimulated by the addition of two different electron acceptors, air and NO_3^-. In the bioaugmentation assays the microcosms were inoculated with different pseudomonads known to degrade chlorobenzene under laboratory conditions [21, 25]. To monitor the fate of the introduced strains, fluorescence *in situ* hybridization (FISH) was used. To detect structure and dynamics of the indigenous communities during the degradation experiments, single-strand conformation polymorphism (SSCP) analysis of 16S rDNA was applied [34]. A total of 224 samples were analyzed and compared using statistical cluster analysis. All community dynamics analysed indicated that rather quickly, community structures appeared that were specific for the chemical conditions imposed or the strains added.

Methods

Site Characterization

The investigated aquifer is located in Bitterfeld, near Leipzig in Saxony, Germany. Contamination of the groundwater extending to an area of about 25 km^2 resulted primarily from chemical industries of the former GDR [47]. The experiments focused on groundwater obtained from the quarternary aquifer in a depth of 17 m. At this depth, the zone adjacent to a lignite layer adsorbing chlorobenzene, the highest contamination was observed. The main contaminants in this aquifer were chlorobenzene (CB), 1,2-dichlorobenzene (1,2-DCB), and 1,4-dichlorobenzene (1,4-DCB) [27]. Average concentrations of these compounds and physicochemical parameters of the groundwater are listed in Table 1.

Bacterial Strains

Pseudomonas aeruginosa RHO1 [25] was isolated based on its capability to mineralize 1,4-dichlorobenzene, but was also reported to be capable of mineralizing chlorobenzene. *Pseudomonas putida* GJ31 mineralizes chlorobenzene via chlorocatechol and a new modified *meta*-cleavage pathway involving a chlorocatechol-2,3-dioxygenase [21]. *Pseudomonas putida* F1ΔCC [20] is a derivative of *Pseudomonas putida* F1 [49] obtained after chromosomal insertion of the *Pseudomonas* sp. strain P51 de-

Table 1. Physicochemical parameters of groundwater of the quaternary Bitterfeld aquifer at 17–20 m depth

Parameter		References
Temperature	14.6°C	GFE[a]
pH	6.94	GFE[a]
0_2	0.2 to 0.4 mg L^{-1}	GFE[a]
Redox potential	−159 to −212 mV	GFE[a]
Conductivity	3.0 mS	GFE[a]
Chlorobenzene	12 to 51 mg L^{-1}	[27]
1,2-Dichlorobenzene	0.07 to 0.18 mg L^{-1}	[27]
1,4-Dichlorobenzene	0.7 to 1.0 mg L^{-1}	[27]
Nonvolatile DOC	10.6 mg L^{-1}	This work
NO_3^-	0 mg L^{-1}	This work

[a] Geologische Forschung und Erkundung GFE GmbH; Bereich Geo-Umwelt-Technik; FG Hydrogeologie/Hydrologie; Köthener Str. 34; D-06118 Halle.

rived *tcbCDEF* genes, under control of the *tcbR* positive regulator. This phenotype results from the combined action of chromosomal encoded *tod*-genes (transformation of chlorobenzene into 3-chlorocatechol [49]) and new integrated *tcb*-genes (transformation of 3-chlorocatechol into metabolites of the Krebs cycle [41]).

All strains were grown in liquid mineral medium [12] with chlorobenzene vapors (corresponding to a concentration of 1–3 mM in total) as sole source of carbon and energy at 30°C. Growth rates in liquid culture were measured by determination of optical density (A_{546nm}), total cell counts (see below), and protein content [5]. The cells were harvested during late exponential growth phase, washed twice in phosphate buffer (24 mM, pH 7.2), and stored at 4°C for 48 h until the experiments were started. Strains were introduced into the groundwater microcosms with initial cell concentrations of 10^5 and 10^6 cells mL^{-1}.

Microcosm Experiments

Biodegradation of chlorobenzenes was assessed in groundwater microcosms [heat-sterilized 1000-mL (nominal volume) glass bottles, Schott, Germany]. Groundwater samples were taken on-site from a well installed in a depth of 17 m to sample natural groundwater. Twenty L of groundwater were mixed to get a homogenous sample. After sampling oxygen, introduced during sampling, was removed by sparging with nitrogen reaching an oxygen concentration of 0.1–0.4 mL^{-1}. This resulted in a loss of the main volatile contaminants. Therefore, the groundwater was spiked with CB (30 mg L^{-1} final concentration), 1,2-DCB (2 mg L^{-1} final concentration), and 1,4-DCB (2 mg L^{-1} final concentration) injected directly into the pooled groundwater. DCB was dissolved in monochlorobenzene (MCB) before injection. The bottles were sealed with Teflon-coated silicon septa in the screw caps. All variants of the different treatments were performed in triplicate. Glass bottles were incubated at 13°C without shaking. The volume of the groundwater at the beginning of the experiments was 800 mL. During biostimulation experiments the potential of the indigenous microbial community was analyzed under anaerobic denitrifying and aerobic conditions. NO_3^- was added at an initial concentration of 2.14 mM in the anaerobic assay. Initial oxygen concentration in the aerobic assays was 2 mg L^{-1}. To check for abiotic losses of chlorobenzenes during the experiments, sterile controls were performed in triplicate. For these controls groundwater was sterilized by a pressure filtration unit (Sartorius; Göttingen, Germany) with nitrogen at a pressure of 200 mbar through a sandwich of a precombusted glass fiber filter (Whatman Corp., diameter 144 mm; type GF/F) on top of a membrane filter (Nuclepore Corp.; diameter, 144 mm; pore size, 0.1 μm).

Chemical Analysis

Depl etion of CB, 1,2-DCB and 1,4-DCB during the course of the experiment was determined by reversed-phase HPLC (Injector Waters 712 WISP; Waters 510 HPLC pump; Waters 996 PDAD, detection at 217 nm) with a Nucleosil 120-C8 5-μm column (250 by 4.6 mm). The mobile phase consisted of 0.1% H_3PO_4 and 70% (v/v) methanol in water. The flow rate was 1 mL min^{-1}. Groundwater samples were taken with gastight syringes (SGE, Weiterstadt, Germany) through the septa of the flasks, placed in evacuated HPLC vials, and subsequently stored at −22°C until analysis. The detection limit for CB under the conditions described was below 1 mg L^{-1}.

Nonpurgable Organic Carbon

To determine the concentration of nonvolatile dissolved organic carbon, the groundwater was filtered through a filter sandwich consisting of a polycarbonate filter (0.2 μm pore size; Nuclepore, UK) and a carrier filter (glass fiber filter, Whatman Corp., type GF/F), pretreated by heating at 450°C for 4 h. All glass instruments were pretreated by heating at 180°C for 4 h to remove volatile carbon. Filtered groundwater was diluted tenfold with Milli-Q water and acidified by the addition of HCl to obtain a pH of 2. Volatile hydrocarbons, including CO_2, were blown out by sparging groundwater with a stream of N_2 for 5 min. Subsequently, nonpurgeable organic carbon was measured by combustion and IR detection using a TOC 5000A total organic carbon analyzer (Shimadzu, Duisburg, Germany).

Total Cell Counts

Groundwater samples were taken with sterile syringes and fixed with formaldehyde (final concentration of 4%). Aliquots of 0.1–1 mL were filtered through a 25-mm diameter filter sandwich consisting of an Anodisc 25 (0.2 μm pore size, Whatman) and a cellulose-nitrate (pore size 0.45 μm, Sartorius, Germany) filter. Cells were stained by the nucleic acid dye SYBR Green I (Molecular Probes) as described by Weinbauer et al. [46] and enumerated using an epifluorescence microscope (Axiophot 135 TV, filter 09, Zeiss, Germany). At least 20 × 200 cells from chosen viewing fields were counted.

Table 2. 16S rRNA primers and oligonucleotides for FISH analysis

Target group	Probe sequence 5′ to 3′	Probe name [reference]
Domain Bacteria	AGG CCC GGG AAC GTA TTC AC	13B [31]
Species *P. putida*	ACT GCA TCC AAA ACT GGC AA	Pp 16S [14]
Species *P. aeruginosa*	TGC ATC CAA AAC TAC TGA GCT	Pa16S [this work]
Species *P. putida*	TTG CCA GTT TTG GAT GCA GT	Pp16-R [14]
Species *P. aeruginosa*	AGC TCA GTA GTT TTG GAT GCA	Pa16S-R [this work]
Domain Bacteria	CAG CAG CCG CGG TAA TAC	F-Com1 [34]
Domain Bacteria	CCG TCA ATT CCT TTG AGT TT	R-Com2-Ph [34]

Monitoring of Inoculated Strains

P. putida strains GJ31 and F1ΔCC and *P. aeruginosa* RHO1 were detected by fluorescence *in situ* hybridization (FISH) with probes that hybridize within the V4 variable region of the 16S rRNA. For detection of *P. putida* strains the probe Pp16S-R [14] was used, whereas strain *P. aeruginosa* RHO1 was detected with the probe Pa16S-R (Table 2). For this probe the region between the bases 619 and 640 of the 16S rDNA sequence of *P. aeruginosa* was chosen. Probes were synthesized by TIB MOLBIOL (Berlin, Germany) and labeled at the 5′-end with the fluorescent dye Cy3 (Molecular Probes). Twenty-μL to 30-μL aliquots of formaldehyde fixed (final concentration 4%) groundwater were dropped onto glass slides. Cell immobilization and hybridization on slides were performed as described by Amann et al. [2]. Hybridization conditions of Pp16S-R was 46°C for 1.5 h followed by a washing step of 20 min at 46°C. Conditions for hybridization with Pa16S-R were 40°C for 1.5 h and washing at 40°C for 20 min. The concentration of the probes in the hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl pH 8.0, 0.1% SDS) were 6 and 8 ng μL^{-1} for Pa16s-R and Pp16S, respectively. Staining for total cell counts was carried out with DAPI (5.5 μg μL^{-1}, Sigma, Germany) for 5 min. Slides were mounted with the antifade solution Citifluor glycerol/PBS (Plannet GmbH, Germany) and cells were enumerated by epifluorescence microscopy (Axiophot 135 TV, Zeiss, Germany; filter 09, Zeiss, Germany; Filter HQCy3, AF Analysentechnik, Tubingen, Germany). At least 200 cells from 20 fields of view were counted. A cross-reaction of the labeled FISH probes with the indigenous bacteria was not observed.

Sampling of Groundwater during the Microcosm Experiments and DNA Extraction

Fifty mL of water was drawn with sterile syringes through the septum during each sampling. Anaerobic samples were taken with parallel introduction of nitrogen. Bacteria were collected by filtration through a 0.22 μm-pore-size Durapore filter (Sartorius, Göttingen, Germany), 47 mm in diameter. Filters were stored at −20°C until DNA extraction. The DNA extraction procedure of Fuhrman et al. [16] was modified as follows. Frozen filters were thawed for extraction of DNA, cut with a clean razor blade into small strips, and vortexed briefly in 1 mL STE buffer [10 mM Tris hydrochloride (pH 8.0), 1 mM EDTA, 100 mM NaCl, and 1% sodium dodecyl sulfate] in reaction tubes (2 mL volume; Eppendorf, Hamburg, Germany). These tubes were incubated at 95°C for 2 min. Samples were cooled down on ice. Cellular debris and filter residues were pelleted by centrifugation (10 min at 10,000 *g*) at 4°C. The liquid phase was transferred into a fresh tube and mixed with 1 volume of phenol (pH 8.0) saturated with TE buffer (10 mM Tris base, 1 mM Na_2-EDTA; pH 8.0). The suspensions were then centrifuged as described above. The upper phase was transferred in a fresh tube and mixed with an equal volume of chloroform/isoamylalcohol (24:1, vol/vol). The suspensions were centrifuged again (see above). The upper phase was transferred into a fresh tube (2 mL volume). DNA was precipitated with 1 volume isopropanol and 0.1 volume sodium acetate (3 M) at −20°C for at least 1 h. DNA was collected by centrifugation at 15,000 *g* for 30 min at 4°C. Pellets were washed with 70% ethanol, dried at room temperature, and resuspended in TE (pH 8.0).

PCR Amplification and SSCP Analysis

PCR amplification of 16S rDNA from the extracted DNA and SSCP analysis were done according to Schwieger and Tebbe [34]. Briefly, after PCR amplification of a part of the 16S rRNA gene (primer set Com 1; positions 519 to 536 and Com2-Ph; positions 907 to 926, see Table 2), the phosphorylated strand of the amplicon was removed by λ-Exonuclease digest. To introduce specific secondary structures in the strands, samples were heat denatured and cooled on ice and then were loaded onto a denaturing gel. Gels were silver stained according to the procedure of Bassam et al. [3].

SSCP Fingerprint Analysis and Statistics

All microcosm experiments were run as triplicates for each assay. Therefore, interpretation of SSCP results in this study was based on evaluation of three independent microcosms and gel runs. For cluster analysis the gels were digitalized to create TIFF files. Cluster analysis of the 16S rDNA fingerprints was performed using the software package GelCompare II (Applied Maths, Kortrijk, Belgium). Background was first subtracted using rolling circle correction (circle diameter, 30 pts) and lanes were normalized. Only bands with an intensity of 2% or more of the total lane intensity were considered for the statistical analysis. Dendrograms were constructed based on gel scans of each fin-

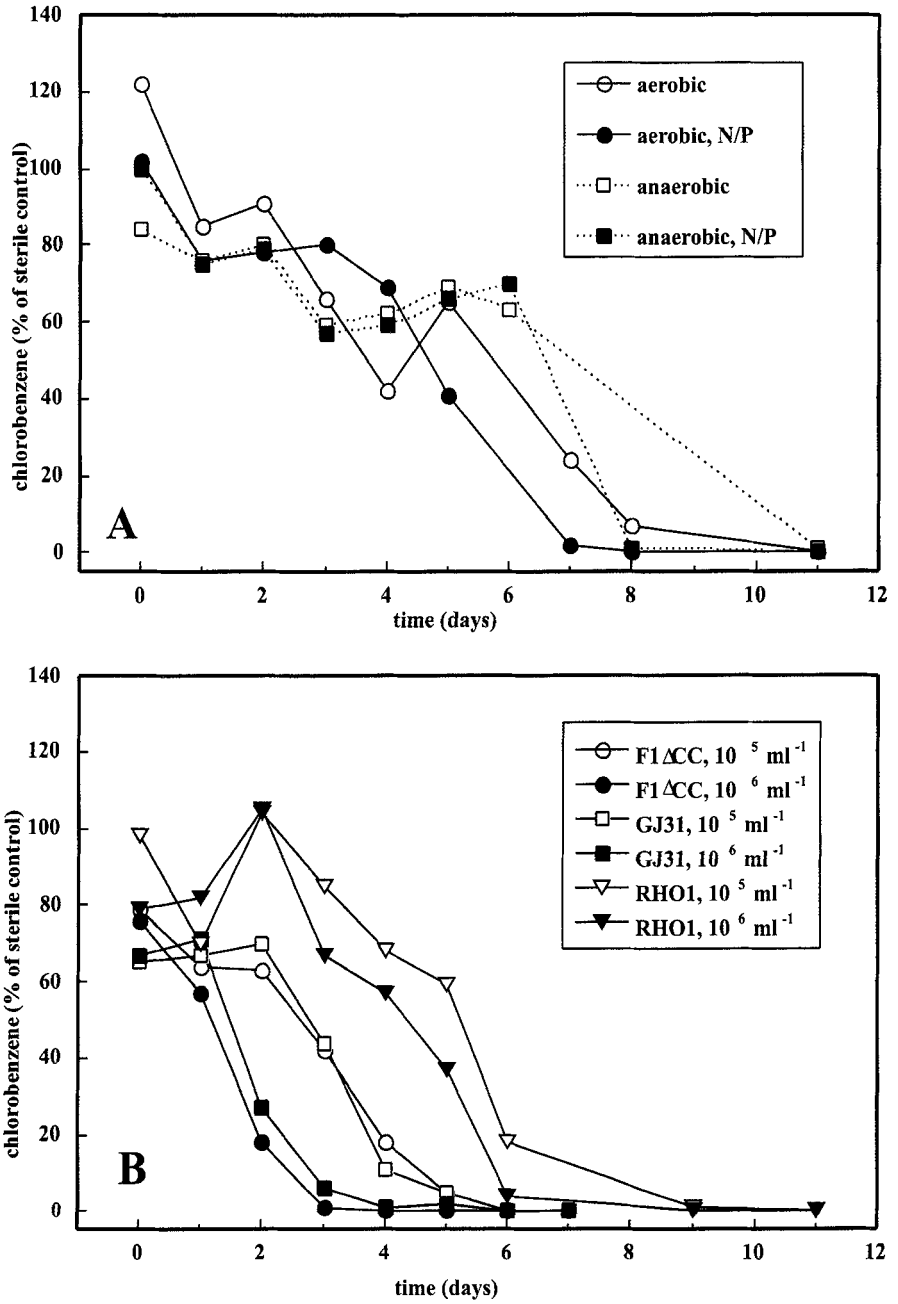

Fig. 1. Chlorobenzene depletion in microcosms with groundwater originating from the aquifer of Bitterfeld. (A) Biostimulation experiments: Groundwater was supplemented with oxygen (aerobic, corresponding to 3.5 mM) or nitrate (anaerobic, 2.14 mM NO_3^-) as terminal electron acceptor. Experiments were performed using groundwater with no further supplementation or receiving 0.1 mM NH_4^+ and 0.025 mM PO_4^{3-} (indicated as N/P). (B) Bioaugmentation experiments: Groundwater was supplemented with oxygen and inoculated with *Pseudomonas putida* F1ΔCC, *Pseudomonas putida* GJ31, or *Pseudomonas aeruginosa* RHOl at initial densities of 10^5 cells mL^{-1} or 10^6 cells mL^{-1}, respectively. The chlorobenzene concentration (initially 30 mg L^{-1}) is given as percentage of the sterile control.

gerprint by using the Jaccard coefficient [35] and the neighbor-joining clustering method. The Jaccard coefficient considers only the peak position in the gel scans. Before calculation of the dendrograms dealing with the three introduced strains, the specific bands of each of the strains were deleted.

Results

Biostimulation Experiments

Chlorobenzene concentrations were rather stable in the sterile controls and are given as percentage of these sterile controls for better comparability. Monochlorobenzene concentrations decreased in groundwater microcosms containing oxygen or nitrate as supplemented final electron acceptors, during the course of the experiments (Fig. 1A). A similar decrease was observed for 1,2- and 1,4-dichlorobenzenes (data not shown). No residual chlorobenzene could be detected in any of the biostimulation experiments after an incubation period of 11 days, indicating the potential of the natural microbial community to transform chlorobenzenes under any of the given conditions. Nitrate concentration in the anaerobically incubated groundwater decreased by more than 1 mM during the course of the experiment (data not shown). This could indicate the use of NO_3^- as electron acceptor in the process of CB degradation.

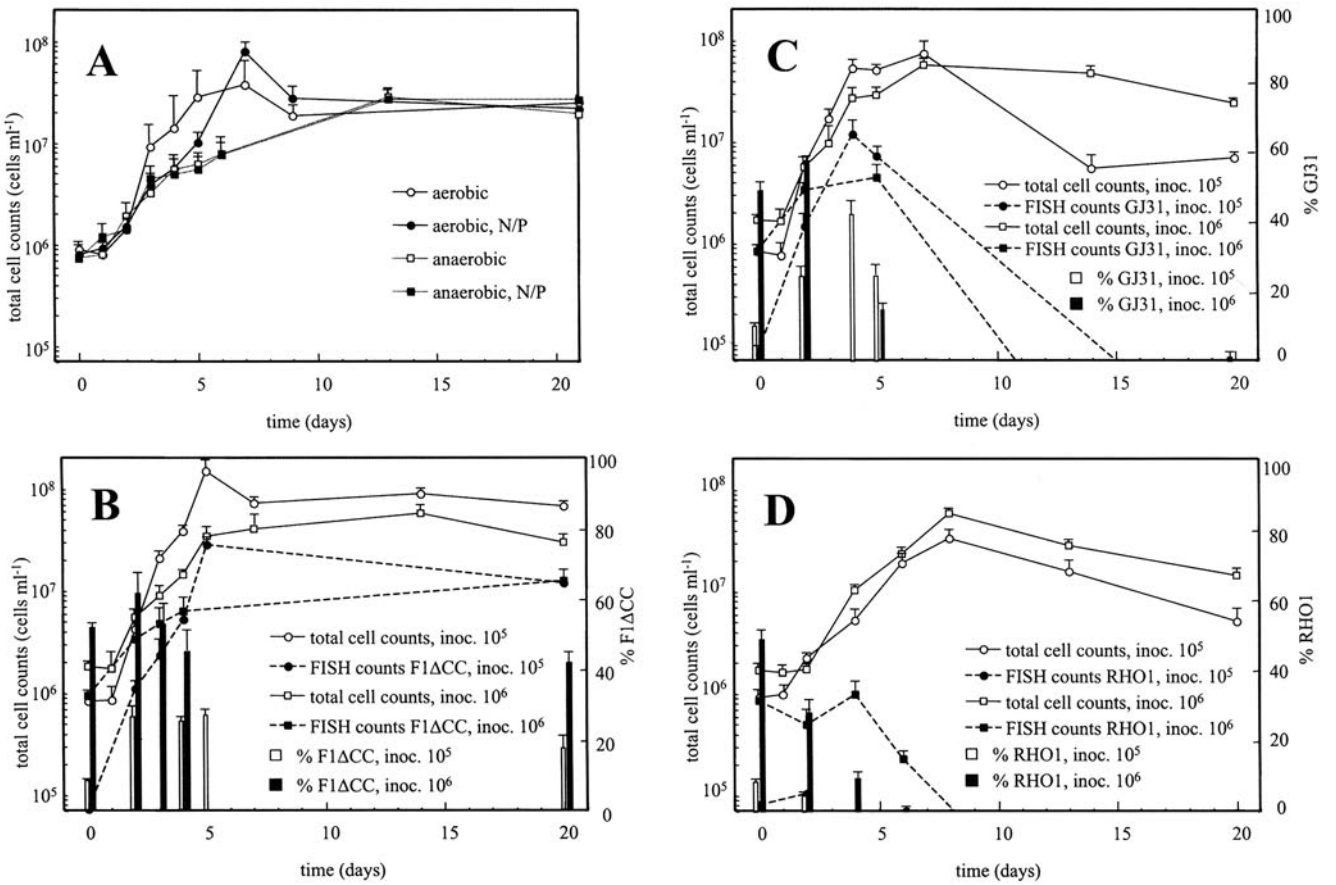

Fig. 2. Population dynamics of indigenous and inoculated microorganisms in groundwater microcosms. (A) Total bacterial cell numbers in microcosms supplemented with oxygen (aerobic) or nitrate (anaerobic) as terminal electron acceptor. N/P indicates experiments, which received further supplementation by NH_4^+ (0.1 mM) and PO_4^{3-} (0.025 mM). (B) Total bacterial cell numbers, cell numbers of *P. putida* F1ΔCC, and percentage of F1ΔCC with respect tothe total cell number (given in bars) in microcosms inoculated with *P. putida* F1ΔCC at initial densities of 10^5 cells mL^{-1} or 10^6 cells mL^{-1}, respectively. (C) Total bacterial cell numbers, cell numbers of P. *putida* GJ31 and percentage of GJ31 with respect to the total cell number (given in bars) in microcosms inoculated with *P. putida* GJ31 at initial densities of 10^5 cells mL^{-1} or 10^6 cells mL^{-1}, respectively. (D) Total bacterial cell numbers, cell numbers of *P. aeruginosa* RHO1, and percentage of RHO1 with respect to the total cell number (given in bars) in microcosms inoculated with *P. aeruginosa* RHO1 at initial densities of 10^5 cells mL^{-1} or 10^6 cells mL^{-1}, respectively. (A)–(D) All data points show mean values and standard deviation from triplicate microcosms. Bioaugmentation experiments using initial cell densities of 10^5 cells mL^{-1} are indicated as "inoc.10^5" those with initial cell densities of 10^6 cells mL^{-1} are shown as "inoc. 10^6".

The total bacterial cell numbers in the groundwater microcosms during the course of the biostimulation experiments increased significantly after a lag phase of 1 day (Fig. 2A) from values of 8×10^5 cells mL^{-1}, as observed in the untreated native groundwater, to cell numbers higher than 10^7 mL^{-1} after 7 days. During this growth phase growth rates were observed in the range of 0.7–0.9 d^{-1} (corresponding to doubling times of 18–24 h) under aerobic conditions and 0.4–0.5 d^{-1} (doubling times 33–42 h) under anaerobic conditions. After this growth phase of about 7 days a stationary phase was reached from day 9 on, corresponding with the complete depletion of the chlorobenzenes.

The analysis of the effect of different electron acceptors on the community structure and dynamics of the bacterial community in the microcosms consisted of three steps: (i) all samples were analyzed by SSCP fingerprinting on native polyacrylamide gels, (ii) all banding patterns were normalized against a universal standard to allow comparison across different gels, and (iii) all runs were compared by cluster analysis. The cluster analysis of SSCP fingerprints from the biostimulation experiments is shown

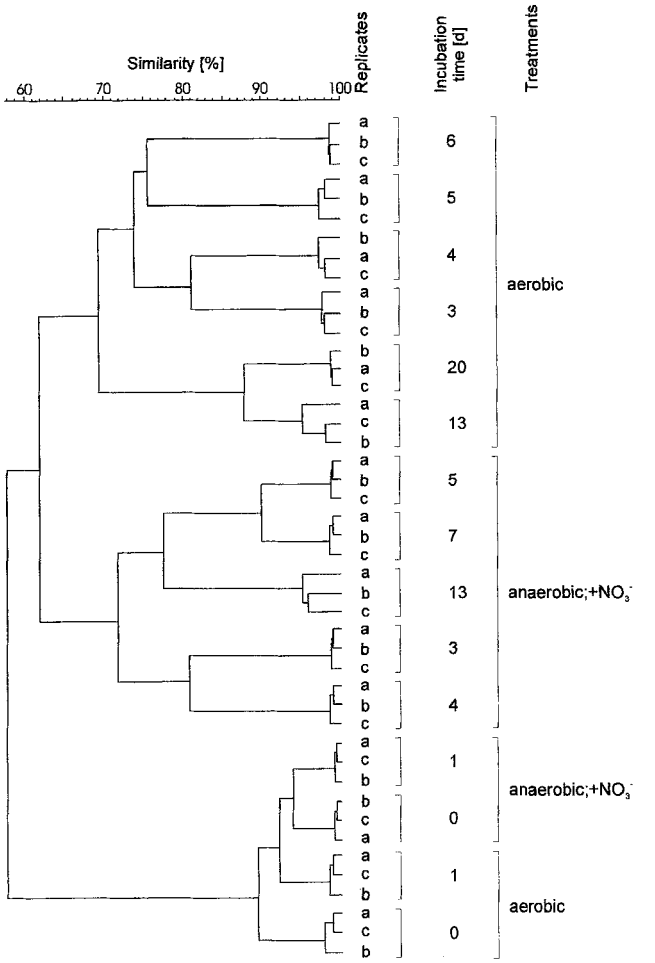

Fig. 3. Cluster analysis of SSCP community fingerprints from the biostimulation experiments based on positions of bands using unweighted pair grouping with Jaccard coefficient.

in Fig. 3. Three main clusters are observed: (1) communities at the beginning of the experiment (0 and 1 day), (ii) communities incubated under denitrifying conditions (3–13 days), and (iii) aerobically incubated communities (3–20 days). Each sample of the triplicate microcosms with the same treatment and taken at the same time showed a very similar fingerprint indicated by the high similarities among the triplicates (>95% in Fig. 3). This demonstrates the high reproducibility of the results obtained from three independent microcosms and resulted in very similar community dynamics of the single microcosms. During incubation, two different communities have been selected as shown by two main clusters. Within each cluster, similarities of about 50% were found. Obviously, the bacterial communities after 3 and 4 days, 5 and 6 days, and 13 and 20 days incubation time for both experiments grouped together. In correspondence to the changes in community structure, the total bacterial cell numbers increased under the different incubation conditions (Fig. 2A), i.e., major changes occurred in the growth phase and only minor changes were observed in the stationary phase.

For a better understanding of the community dynamics, the total number of bands in the SSCP fingerprints, reflecting species richness of the community, was assessed (Fig. 4A). After the lag phase, the number of bands increased more than threefold during the growth phase with both electron acceptors. Whereas in the aerobic microcosms the richness remained high after the fourth day, it dropped by a factor of 2 in the denitrifying experiments after this day and slowly increased during the rest of the experiment.

Bioaugmentation Experiments

Different effects on the chlorobenzene concentrations were observed after inoculating the microcosms with each of the three bacterial strains (Fig. 1B). Bioaugmentation with *P. putida* F1ΔCC or GJ31 significantly increased the degradation rate. Inoculation at high cell densities corresponding to those observed in the original groundwater (10^6 autochthonous cells mL^{-1}) resulted in a rapid chlorobenzene depletion, and no residual chlorobenzene could be detected after an incubation period of 4 days. Inoculation with lower cell numbers (10^5 cells mL^{-1}) showed no significant difference during the first 2 days in CB depletion. However, significant CB depletion due to this inoculum was not expected, as the degradation capability of 10^5 cells mL^{-1} as deduced from transformation experiments using resting cells can be calculated to be in the range of 1 μM of chlorobenzene per day. The fast chlorobenzene depletion during the further time course of the experiment (day 3–5), which was enhanced over that observed in biostimulation experiments, must thus be assumed to be due to rapid growth of the inoculated organisms in the groundwater microcosms.

Bioaugmentation with strain RHO1 had only a negligible effect on chlorobenzene transformation when compared with biostimulation or bioaugmentation experiments using *P. putida* strains F1ΔCC or GJ31 (Fig. 1B). Chlorobenzene was depleted only after an incubation period of 9 days. Slightly lower chlorobenzene concentrations during the incubation period were observed in microcosms inoculated with 10^6 cells mL^{-1} compared to those inoculated with 10^5 cells mL^{-1}, indicative of some activity of the introduced strain on the chlorobenzene degrading performance in the microcosms.

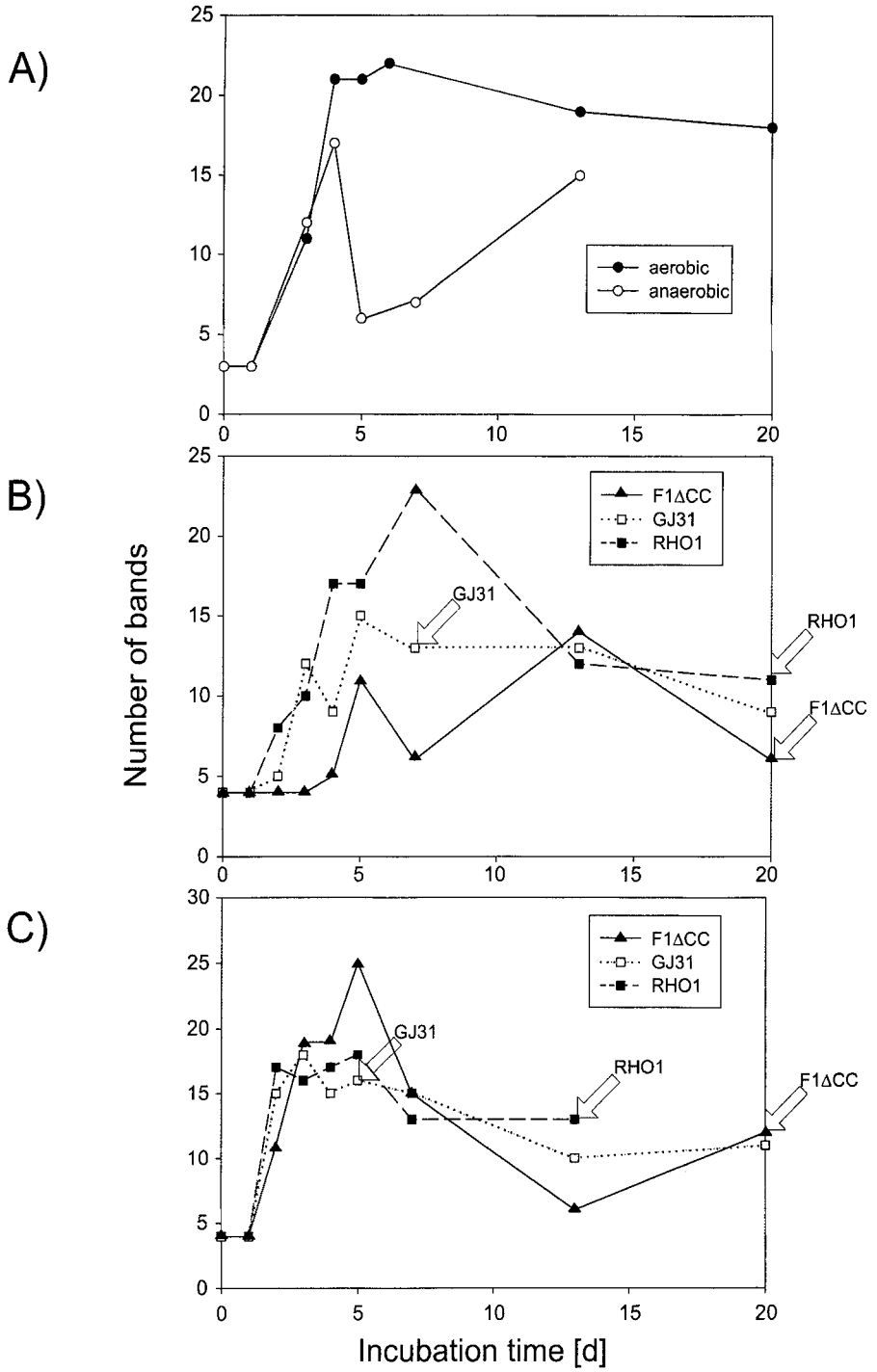

Fig. 4. Total number of significant bands per SSCP fingerprint during biostimulation (A), and bioaugmentation with initial cell densities of 10^5 cells mL^{-1} (B) and 10^6 cells mL^{-1} (C), respectively. Only bands above the threshold of more than 2% of the total peak area were counted as significant. The arrows point to the incubation time when the introduced strains could be detected the last time by PCR-SSCP.

Corresponding to the chlorobenzene depletion, changes were observed in the total number of bacterial cells and the specific cell number of the introduced strains (Figs. 2B–D). The curves reflect the total cell numbers in the bioaugmentation assays and the cell numbers of the introduced strain as measured by FISH analysis. The bars represent the percentage of the inoculated strain within the autochthonous community at distinct time points. In assays inoculated with F1ΔCC or GJ31 at initial cell densities of 10^5, corresponding to about 10% of the indigenous bacterial community, growth of inoculants occurred at rates of μ = 1.1–1.3 d^{-1}, corresponding to doubling times of 13–15 h. Those growth rates were similar to the one observed for F1ΔCC in filter-sterilized groundwater in the presence of chlorobenzene (1.4 d^{-1}, data not shown). The inoculated strains F1ΔCC and GJ31 reached cell numbers of more than 10^7 mL^{-1} in between 4–5 days of incubation and percentages of up to 30–60% of the total cell numbers.

SSCP fingerprints from all different assays were performed to assess the influence of the introduced strains

with two densities different by an order of magnitude on the indigenous bacterial communities. Figure 5 shows a representative silver stained SSCP gel with 16S rDNA fingerprints obtained from the assays inoculated with *P. aeruginosa* RHO1 at an initial density of 10^5 cells mL^{-1} groundwater. The arrow in Fig. 5 points to the specific band of RHO1. All three introduced strains could be detected in the microcosms by SSCP analysis by comparison with SSCP analyses from pure cultures. Strain RHO1 and F1ΔCC were detected during the whole incubation time by FISH and SSCP analysis (Figs. 2B, 4). In contrast, GJ31 was not detected after 7 days (Figs. 2C, D; 4B, C).

The influence of the inoculated strains on the indigenous communities can be seen on the dendrogram resulting from cluster analysis of the SSCP fingerprints with 10^5 cells mL^{-1} inoculum, calculated without the bands of the introduced strains, which shows four different groups (Fig. 6). During the first 24 h of the experiments all samples were highly similar. After 2 days, each microcosm which had received a specific inoculum started to develop a different microbial community which was clearly separated from that of the other experiments and from the first 24 h of the experiments. Also, clear groupings according to growth and stationary phase can be seen in the three strain-specific clusters. Furthermore, during incubation the total number of bands, as assessed by SSCP fingerprints, increased as observed in biostimulation experiments (Fig. 4B).

A second inoculum concentration (10^6 cells mL^{-1}) was used to assess the influence of the initial cell concentration on the degradation kinetics and the community structure of the indigenous bacteria. Significantly faster depletion of chlorobenzene during the incubation period was observed in microcosms inoculated with 10^6 cells mL^{-1} of strain GJ31 and F1ΔCC compared to those inoculated with 10^5 cells mL^{-1} (Fig. 1B). One effect was that the total cell number was higher compared to cell number in the microcosms which were inoculated with 10^5 cells of F1ΔCC and GJ31 (Fig. 2B, C). The highest cell number between among microcosms inoculated with 10^6 cells was found in the assay with RHO1 (5×10^7 cells mL^{-1}). Figure 7 shows band patterns of the 16S rDNA amplicons from the experiment with the introduction of RHO1. This strain persisted during the second experiment only for a short time as assessed by FISH (Fig. 2D). In contrast to strain F1ΔCC and GJ31, strain RHO1 did not grown in any microcosms, but cell numbers as estimated by FISH analysis remained constant for 2–4 days and declined thereafter.

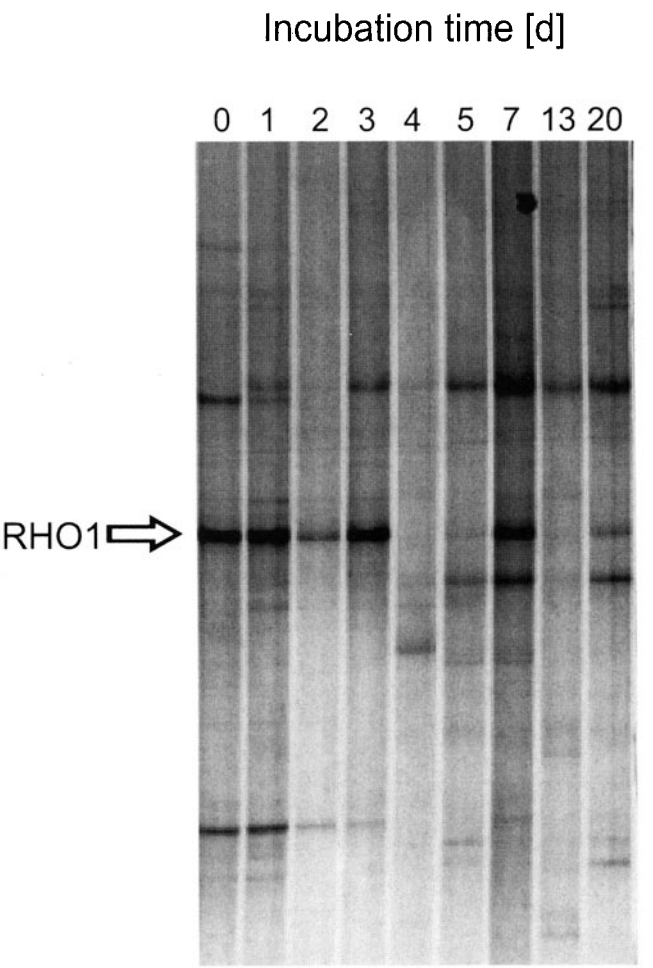

Fig. 5. Comparison of SSCP fingerprints from bacterial communities in groundwater microcosm experiments inoculated with strain RHO1 with initial cell density of 10^5 cell mL^{-1}. Arrow points to the specific band position of RHO1 as known from pure cultures.

Whereas F1ΔCC and GJ31 behaved similarly in the presence of chlorobenzene, i.e., growth and enhanced chlorobenzene removal, significant differences were observed after chlorobenzene depletion. Strain F1ΔCC persisted in the groundwater during GJ31 declined (Fig. 2C). After 20 days incubation only a few *P. putida* GJ31 cells could detected by FISH.

The cluster analysis based on the SSCP fingerprints (Fig. 8) revealed four major groups. After 1 day incubation each bioaugmentation experiment developed its own community induced by the presence of the introduced strain. It is noteworthy, that two fingerprints out of the GJ31 assay (<13 days) clustered in the group of the fingerprints taken after early incubation times (1 day or below). This could indicate that toward the end of this experiment the community structure resembled to the one

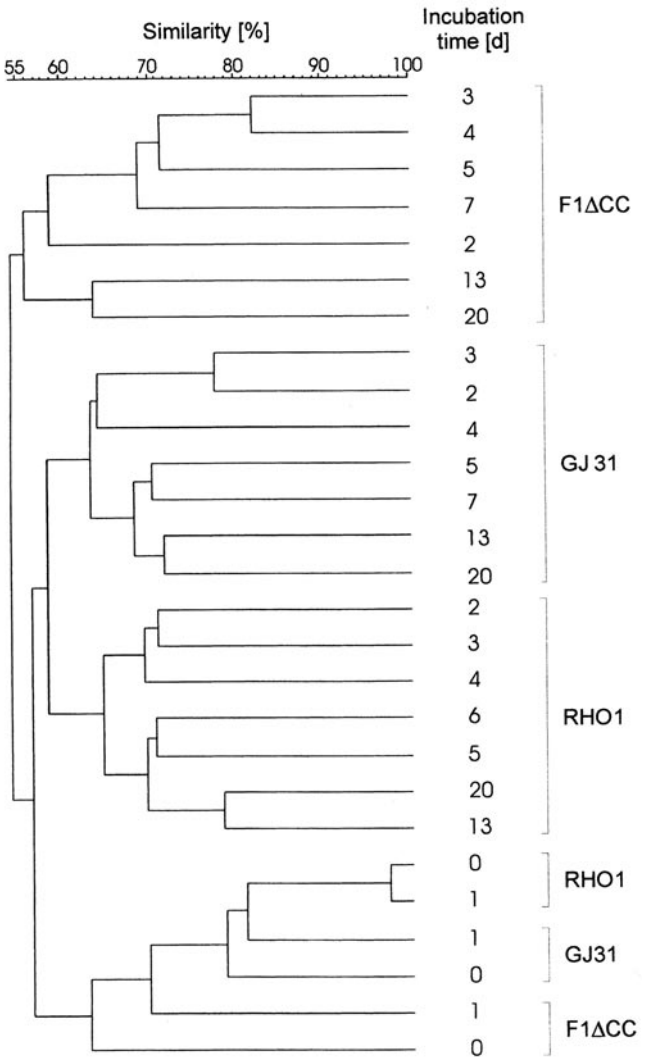

Fig. 6. Cluster analysis of SSCP fingerprints based on the positions of bands using unweighted pair grouping of the Jaccard coefficient, obtained from bands originating from community DNA isolated during bioaugmentation experiments (inoculum size 10^5 cells mL^{-1}). To compare the indigenous bacterial community only, bands originating from introduced strains were not included in the cluster analysis.

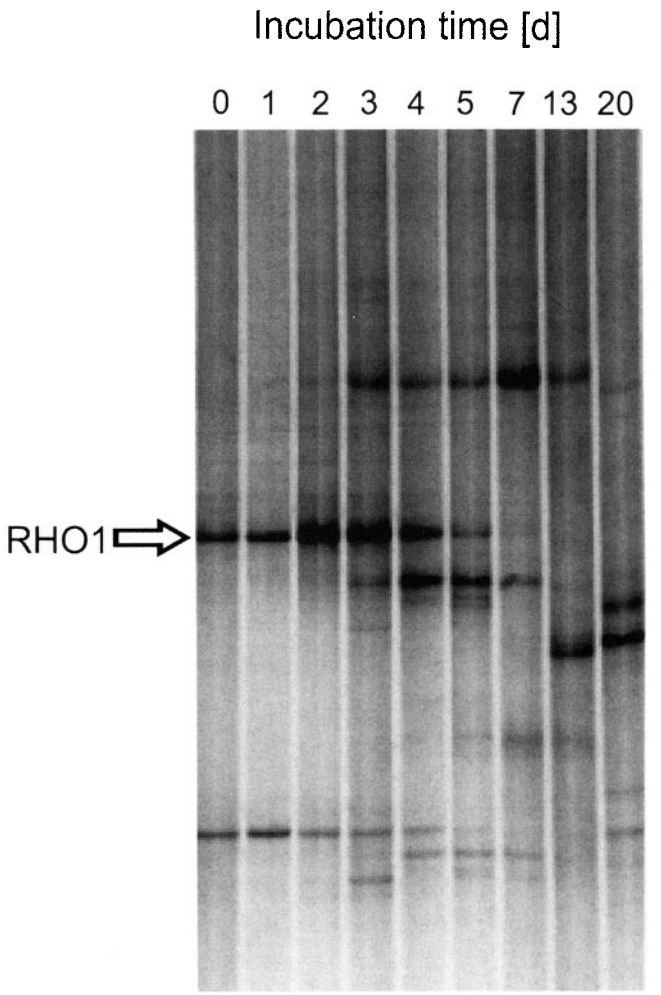

Fig. 7. Comparison of SSCP patterns from bacterial communities in groundwater microcosm experiments inoculated with strain RHO1 with initial cell density of 10^6 cell mL^{-1}. Arrow points to the specific band position of RHO1.

at the beginning. In all these experiments an increase in band number was observed after 2 days of incubation (Fig. 4C). The highest number of bands (25) was detected in the assay inoculated with F1ΔCC after 5 days.

Finally, a cluster analysis of all bioaugmentation assays was done to compare the effects of inoculation size and strain specificity on the community structure of the indigenous bacterial communities (Fig. 9). The same division into four main clusters was observed as for the single inoculum analysis, i.e., the three different strains and the starter communities at day 0 and 1. The two different inocula resulted in subgroups within the main grouping of the specific strains, i.e., the influence of the specific strains is more important than the size of its inoculum.

Discussion

Chlorobenzene Degradation and Community Dynamics in Biostimulation Experiments

Natural attenuation can be an important process by which pollutants disappear from the environment without high treatment costs. This process is suitable for natural chemicals, such as petroleum hydrocarbons, which are readily metabolized by indigenous microorganisms in most ecosystems [42]. However, chlorinated aromatics such as chlorobenzenes are usually not readily metabo-

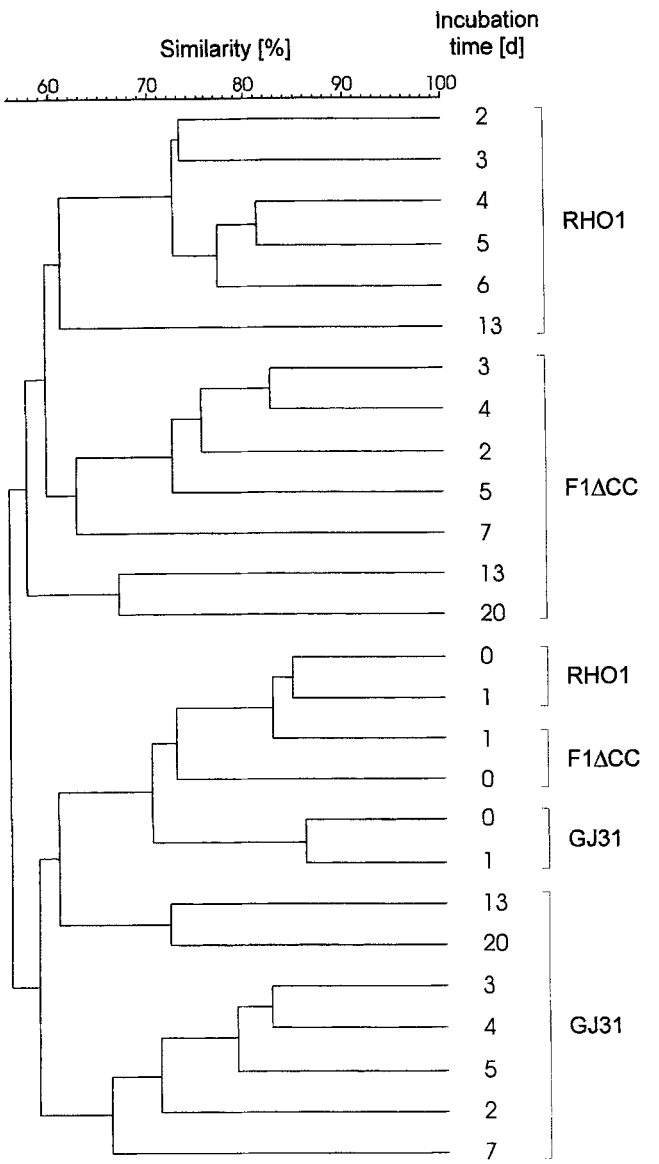

Fig. 8. Cluster analysis of SSCP fingerprint patterns based on the positions of bands using unweighted pair grouping of Jaccard coefficient, obtained from bands originating from community DNA isolated during bioaugmentation experiments (inoculum size 10^6 cells mL^{-1}). To compare the indigenous bacterial community only, bands originating from introduced strains were not included in the cluster analysis.

lized by natural microbial communities [18, 30, 42]. Such recalcitrance under natural conditions can be due to either a missing degradative potential. or a missing bioavailability, or due to unfavorable environmental conditions such as restriction in the availability of nutrients, electron donors, and/or electron acceptors. To overcome these environmental restrictions, *in situ* bioremediations commonly rely on a substantial external supply of organic substrates, additional nutrients, and/or oxygen. For example, aerobic degradation of chlorobenzene has been described in various cases [18, 24, 25, 43].

The most dominant anaerobic degradative route for organohalogens is reductive dehalogenation [19]. Such a process has often been observed in the metabolism of TCE [17, 23], and additionally various halophenol reductive dehalogenases [39, 40] have been described. Chlorobenzene reductive dehalogenation has thus far usually been observed as a fortuitous process [28], and only recently has a first culture capable of reductive chlorobenzene degradation been described [1]. However, only dehalogenation of highly chlorinated benzenes into lower chlorinated ones has been reported. Mineralization of chlorobenzenes in the presence of anaerobic conditions or additional nitrate as electron acceptor has thus far not been described. Our results indicated that the indigenous community had the potential to degrade chlorobenzene if an appropriate electron acceptor such as nitrate was added. The oxygen concentration in the anaerobic bioaugmentation experiments was adjusted to 0.1–0.4 mg L^{-1} groundwater. This amount of oxygen (lower than 12 μM) was not enough to explain chlorobenzene degradation (more than 250 μM) or even the activation (dioxygenation) in the presence of nitrate just based on usage of residual oxygen. As an anaerobic chlorobenzene degradation potential is assumed not to be ubiquitous, it was surprising to observe a respective chlorobenzene disappearance in reasonably short incubation periods concomitant with an increase in cell number. It should be noted that besides small cells, in biostimulation experiments large cells, resembling in size those added in bioaugmentation experiments, were observed by microscopy, indicating mineralization of the substrate (data not shown).

The addition of the electron acceptors (NO_3^- and oxygen) resulted in different bacterial communities as indicated by different positions of the treatments in the dendrogram (Fig. 3). These shifts in community structure occurred in parallel with an increase in total cell numbers (Fig. 2) and numbers of bands (Fig. 4). After 6 to 13 days of incubation the highest cell numbers in both aerobic and anaerobic assays were observed concomitant with substrate depletion. In these samples the highest number of bands was detected indicating that a complex microbial community became activated. Similar findings were reported by Cho and Kim [9]. They found that a phenanthrene-degrading community changed its structure after stimulation with NO_3^- and PO_4^{3-} within 1 week. The reason

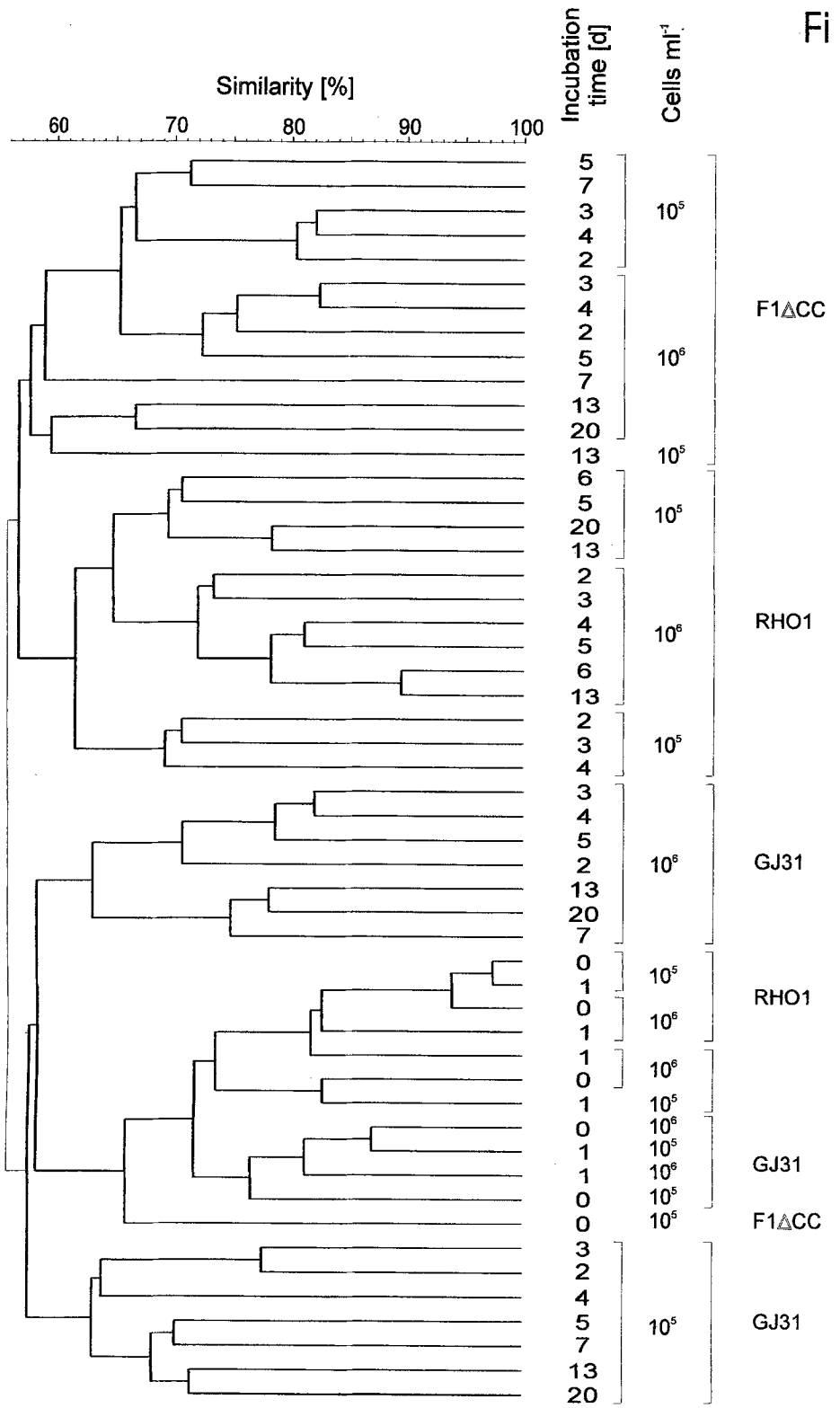

Fig. 9. Dendrogram showing the similarity of the SSCP fingerprints of the indigenous bacterial communities obtained from the microcosm experiments inoculated with the special chlorobenzene degraders at the initial cell densities of 10^5 and 10^6 cells mL^{-1}. Cluster analysis was done using the Jaccard coefficient and unweighted pair grouping.

for the faster changes of our groundwater community could be a better distribution of the added nutrients resulting in a better availability for the bacteria in comparison to soil microcosms. Such a fast change in the structure of a bacterial community after stimulation was also observed by Delbès et al. in a sludge bioreactor [11]. They measured an increase in specific peaks which represented specific taxa by SSCP fingerprinting. In our study addition of electron acceptors resulted in an increase of taxa as indicated by an increase in the total number of bands in the SSCP fingerprints. Other biostimulation studies dealing with bioreactor communities under

anaerobic conditions and substrate mixtures [36] pointed out that stimulation of bacterial communities resulted in a selection of specific bacterial taxa. However, in our study the number of taxa increased as a consequence of the addition of electron acceptors. It might be that a variety of bacteria were able to degrade chlorobenzenes in our groundwater microcosms, as a consequence of adaptation to the pollutant. After addition of the electron acceptors (NO_3^-, oxygen) the pollutants could be used by more taxa in contrast to the start conditions in our microcosm experiments, when only three taxa (bands) were detected. In their experiments, Stoffels et al. [36] added a substrate which was new to the bacteria present. Only a certain part of the bacteria could use this substrate, which resulted in a decrease of detectable taxa.

Chlorobenzene Degradation and Community Dynamics in Bioaugmentation Experiments

The presence of a catabolic potential at the study site enabled us to investigate the effect of bioaugmentation in concurrence with the natural microbial community and the effect of the introduced microorganisms on the composition of the natural microbial community. Out of a collection of chlorobenzene degrading organisms, we selected three strains, which have been demonstrated to be capable of chlorobenzene degradation in culture experiments at *in situ* temperature (13°C). Assessment of the capabilities of such bacteria introduced into environmental systems requires the study of their proliferation and activity. The population dynamics of the introduced strains needs to be assessed by cultivation independent methods to overcome problems frequently encountered with methods such as differential plating [14]. Therefore, all three strains were monitored by FISH and their specific bands in the SSCP fingerprints.

Addition of *P. aeruginosa* strain RHO1 to aquifer microcosms did not enhance biodegradation in contrast to previous investigations on chlorobenzene degradation by RHO1 in soil slurry [7]. Even though this strain had a selective advantage, i.e., presence of chlorobenzene as a growth substrate, and had been demonstrated to grow under the environmental conditions prevailing in the microcosms, it failed to establish itself and proliferate in the presence of the natural microbial community. However, both *P. putida* GJ31 and *P. putida* F1ΔCC were capable of growing in the groundwater microcosms even in the presence of the natural microbial community. Cell densities reached were higher than 10^7 mL^{-1}, indicating that the cells were using the chlorobenzenes added as carbon and energy source and not just transforming this compound. The observed cell densities corresponded well to those reached in batch culture experiments (3×10^7 mL^{-1}/0.25 mM chlorobenzene). In addition to chlorobenzene (30 mg L^{-1}), the aquifer microcosms contained about 10 mg L^{-1} of nonvolatile organic carbon, out of which only a small fraction can be assumed to be readily available [38]. Even assuming a portion of this organic carbon to be biodegradable, it does not explain the high number of cells and cell mass reached after complete chlorobenzene depletion. Moreover, control experiments indicated that *P. putida* F1ΔCC replicated only insignificantly when inoculated into sterile groundwater microcosms with no chlorobenzene added. Good survival of F1ΔCC was also reported by Ravatn et al. [29], who observed that the parental strain *P. putida* F1 survived more than 20 days in a laboratory-scale activated sludge.

Interestingly, the cell numbers of indigenous organisms increased to a similar extent in microcosms supplemented with a specialized strain compared to the biostimulation experiment. However, the cell number alone is not a significant indicator for the increase of biomass. It should be noted that during the bioaugmentation experiments, the indigenous microorganisms kept their small size, thus constituting, compared to the inoculated organism, only a small portion of the total biomass. It can be assumed that the indigenous microbial community in bioaugmentation experiments replicates at the expense of the additional nonvolatile organic carbon and/or excretion products from the inoculated specialists.

A variety of studies have shown that the introduced strains can change the bacterial community. This holds for aquatic as well as terrestrial habitats. For example, Boon et al. [4] reported that introduction of a genetically engineered *Comamonas testosteroni* strain I2 gfp changed the community in a semicontinuous activated sludge reactor as assessed by DGGE. It is obvious that the bioaugmentation treatment stimulated the indigenous bacterial community enough to cause rapid shifts in the community structure after 2 days' incubation (Figs. 4B, C, 6, and 8). Similar findings were reported by Stoffels et al. [36] using the FISH technique. They observed rapid significant shifts in a bioreactor community. De Leij et al. [10] demonstrated that the release of wild-type and recombinant *Pseudomonas fluorescens* strains resulted in significant but transient perturbations of cultivable members of the

indigenous microbial community of the phytosphere of wheat.

In all bioaugmentation assays the number of bands increased with increasing incubation time. However, in the bioaugmentation assays inoculated with 10^5 cells mL^{-1} the highest number of bands was detected after inoculation with strain RHO1, in contrast to the assays inoculated with 10^6 cells mL^{-1} where inoculation with strain F1ΔCC showed the highest band number. It was not clear if the increase in number of bands caused by the introduced strains or the aerobic conditions, because an increase in the band number was detected in the biostimulation experiments as well (Fig. 4A).

For a systematic understanding of community changes induced by specialized allochthonous strains, we performed groundwater microcosm experiments with three different specialists that had the same function, i.e., aerobic degradation of chlorobenzenes, using two different inoculation sizes. The resulting changes of the community structure and dynamics could be clearly analyzed (Fig. 9); after 2 days specific microbial communities occurred in response to the introduced strain. Within these strain-specific community structures the two different inoculation sizes induced their own community structures, thereby indicating that strain specificity is independent of the inoculum size. A variety of reasons could be responsible for these strain-specific community dynamics: (i) different degradation kinetics generating different substrate concentrations, (ii) release of different metabolites, (iii) lysis of strains generating different substrates, (iv) different inhibitory effects by the different strains, and (v) unknown responses to viral lysis or protozoan grazing. These reasons could have functioned singly or in combination.

A general explanation for specific influence of even inoculated strain could be, as pointed out by De Leij et al. [10], that a perturbed environment is characterized by r-strategists and undergoes rapid changes while K-strategists dominate in stable nonperturbed environments. In addition it has to be considered that the groundwater was perturbed by the sampling and the exposure to oxygen. This assumption would explain the low number of taxa detectable by SSCP at the beginning of the experiments. Hence, inoculation of the perturbed groundwater community with the fast-growing *Pseudomonas* strains resulted in different strain-specific communities. The reason for this strain-specific effect might be that the introduced bacteria were active and therefore could inhibit the indigenous bacteria [10].

Both GJ31 and F1ΔCC within the different bioaugmentation experiments were effective CB degraders under the conditions tested, especially considering the added high dose of CB at the beginning of the experiments (Fig. 1B). However, even GJ31 and F1ΔCC, which were similarly effective in CB degradation, generated rather different communities. A possible explanation is that the introduced strains released different exudates that could be utilized by the indigenous bacterial communities for cell maintenance and growth. Among those could be that intermediates of chlorobenzene or dichlorobenzene transformants which can serve as C source for other community members. For example chloroaromatics can be excreted by organisms that have a chlorocatechol-1,2-dioxygenase pathway [26] and the chlorocatechol-2,3-diogenase pathway. In addition, the introduced strains, with the exception of F1ΔCC, died and could be utilized themselves as nutrients by part of the indigenous bacteria in the groundwater (Figs. 3, 4). In conclusion, all the potential reactions of the community had the same effect: the dynamic pattern of interactions during each biostimulation and bioaugmentation assay created a continously changing set of different niches that led to a specific community structure for each assay depending on the introduced strain, its inoculum size, and the imposed physicochemical conditions.

Conclusions

We have tested both strategies, biostimulation and bioaugmentation, for the *in situ* bioremediation of groundwater contaminated with chlorobenzenes. The results indicate that both strategies led to rapid degradation of chlorobenzene despite essentially different community structure of the degrading bacterial assemblage. In the biostimulation assays oxygen as well as nitrate could be used as electron acceptor. Oxygen could be combined with specialized aerobic degraders to obtain the fastest degradation. In conclusion a mixed strategy could be envisaged for an *in situ* bioremediation of the aquifer of Bitterfeld using additional electron and a specialist such as strain GJ31, specially profiting from the finding that the specific strain is more relevant than a large inoculum. The results of this work indicate that it is possible to bioremediate the Bitterfelder groundwater. Both strategies, biostimulation and bioaugmentation, have the disadvantage that they disturbed the indigenous bacterial community. However, this work points out that both strategies are useful tools to remediate a pol-

luted groundwater *in situ*. Understanding those interactions could contribute to the design of better *in situ* bioremediation strategies for contaminated groundwater.

Acknowledgements

The authors are grateful to Julia Bötel and Silke Pretzer for skillful technical assistance and Sven Langener for analysis of chlorobenzenes. The valuable advice on the experimental design by Ingrid Brettar is greatly appreciated. This work was supported by a strategy fund project "System Integrated Environmental Biotechnology" grant of the BMBF and the HGF of Germany (grant number 01 SF 9815).

References

1. Adrian L, Szewzyk U, Wecke J, Gorisch H (2000) Bacterial dehalorespiration with chlorinated benzenes. Nature 408: 580–583
2. Amann R, Ludwig W, Schleifer K-H (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microb Rev 59:143–169
3. Bassam BJ, Caetano AG, Gresshoff PM (1991) Fast and sensitive silver staining of DNA in polyacrylamid gels. Anal Biochem 80:81–84
4. Boon N, Goris J, De Vos P, Verstraete W, Top EM (2000) Bioaugmentation of activated sludge by an indigenous 3-chloroaniline-degrading *Comamonas testosteroni* strain, I2gfp. Appl Environ Microbiol 66:2906–2913
5. Bradford MM (1976) A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
6. Bragg JR, Prince RC, Harner EJ, Atlas RM (1994) Effectiveness of bioremediation for the Exxon Valdez oil spill. Nature 368:413–418
7. Brunsbach TR, Reineke W (1994) Degradation of chlorobenzenes in soil slurry by a specialized organism. Appl Microbiol Biotechnol 42:415–420
8. Bulman TL, Newland M (1993) In situ bioventing of a diesel fuel spill. Hydrol Sci 8:297–308
9. Cho JC, Kim SJ (2000) Computer-assisted PCR-single-strand conformation polymorphism analysis for assessing shift in soil bacterial community structure during bioremidiational treatments. World J Microbiol Biotechnol 16:231–235
10. De Leij F, Sutton E, Whipps J, Fenlon J, Lynch J (1995) Impact of field release of genetically modified *Pseudomonas fluorescens* on indigenous microbial populations of wheat. App Environ Microbiol 61:3443–3453
11. Delbès C, Moletta R, Godon J (2000) Monitoring of activity dynamics of an anaerobic digester bacterial community using 16S rRNA polymerase chain reaction-single-strand conformation polymorphism analysis. Environ Microbiol 2:506–515
12. Dorn E, Hellwig M, Reineke W, Knackmuss H-J (1974) Isolation and characterization of a 3-chlorobenzoate degrading pseudomonad. Arch Microbiol 99:61–70
13. Duba AG, Jackson KJ, Jovanovich MC, Knapp RB, Taylor RT (1996) TCE remediation using in situ, resting-state bioaugmentation. Environ Sci Technol 30:1982–1989
14. DuTeau NM, Rogers JD, Bartholomay CT, Reardon KF (1998) Species-specific oligonucleotides for enumeration of *Pseudomonas putida* F1, *Burkholderia* sp. strain JS150, and *Bacillus subtilis* ATCC 7003 in biodegradation experiments. Appl Environ Microbiol 64:4994–4999
15. Dybas MJ, Barcelona M, Bezborodnikov S, Davies S, Forney L, Heuer H, Kawka O, Mayotte T, Sepúlveda-Torres L, Smalla K, Sneathen M, Tiedje J, Voice T, Wiggert DC, Witt DC, Criddle CS (1998) Pilot-scale evaluation of bioaugmentation for in-situ remediation of carbon tetrachloride-contaminated aquifer. Environ Sci Technol 32:3598–3611
16. Fuhrman JA, Comeau DE, Hagström Å, Chan AM (1988) Extraction from natural planktonic microorganisms of DNA suitable for molecular biological studies. Appl Environ Microbiol 54:1426–1429
17. Gerritse J, Drzyzga O, Kloetstra G, Keijmel M, Wiersum LP, Hutson R, Collins MD, Gottschal JC (1999) Influence of different electron donors and accepters on dehalorespiration of tetrachloroethene by *Desulfitobacterium frappieri* TCE1. Appl Environ Microbiol 65:5212–5221
18. Haigler B, Nishino S, Spain J (1988) Degradation of 1,2-dichlorobenzene by a *Pseudomonas* sp. Appl Environ Microbiol 54:294–301
19. Holliger C, Schumacher W (1994) Reductive dehalogenation as a respiratory process. Antonie van Leeuwenhoek 66:239–246
20. Lehning A (1998) PhD thesis. Technical University, Braunschweig, Germany
21. Mars AE, Kasberg T, Kaschabek SR, van Agteren MH, Janssen DB, Reineke W (1997) Microbial degradation of chloroaromatics: use of the *meta*-cleavage pathway for mineralization of chlorobenzene. J Bacteriol 179:4530–4537
22. Miethling R, Karlson U (1996) Accelerated mineralization of pentachlorophenol in soil upon inoculation with *Mycobacterium chlorophenolicum* PCP1 and *Sphingomonas chlorophenolica* RA2. Appl Environ Microbiol 62:4361–4366
23. Neumann A, Wohlfarth G, Diekert G (1995) Properties of tetrachloroethene and trichloroethene dehalogenase of *Dehalospirillum multivorans*. Arch Microbiol 163:276–281
24. Nishino SF, Spain JC, Belcher LA, Litchfield CD (1992) Chlorobenzene degradation by bacteria isolated from contaminated groundwater. Appl Environ Microbiol 58:1719–1726
25. Oltmanns R, Rast H, Reineke W (1988) Degradation of 1,4-dichlorobenzene by enriched and constructed bacteria. Appl Microbiol Biotechnol 28:609–616
26. Pieper DH, Stadler-Fritzsche K, Engesser K-H, Knackmuss H-J (1993) Metabolism of 2-chloro-4-methylphenoxyacetate

by *Alcaligenes eutrophus* JMP 134. Arch Microbiol 160:169–178
27. Popp P, Möder M (1997) Organische Analytik. UFZ-Bericht, Leipzig
28. Ramanand K, Balba MT, Duffy J (1993) Reductive dehalogenation of chlorinated benzenes and toluenes under methanogenic conditions. Appl Environ Microbiol 59:3266–3272
29. Ravatn R, Zehnder A, van der Meer J (1998) Low-frequency horizontal transfer of an element containing the chlorocatechol degradation genes from *Pseudomonas* sp. strain B13 to *Pseudomonas putida* F1 and to indigenous bacteria in laboratory-scale activated-sludge mircocosms. Appl Environ Microbiol 64:2126–2132
30. Reineke W, Knackmuss H-J (1984) Microbial metabolism of haloaromatics: Isolation and properties of a chlorobenzene-degrading bacterium. Appl Environ Microbiol 47:395–402
31. Relman DA (1993) Universal Bacterial 16S rDNA Amplification and Sequencing. American Society for Microbiology, Washington, DC
32. Ruske R, Hübner J, Böhme O, Falke P (1997) Ergebnisse der geologisch-hydrogeologischen-geotechnischen Standorter kundung. UFZ-Bericht, Leipzig
33. Salanitro JP, Johnson PC, Spinnler GE, Maner PM, Wisniewski HL, Bruce C (2000) Field scale demonstration of enhanced MTBE bioremediation through aquifer bioaugmentation and oxygenation. Environ Sci Technol 34:4152–4162
34. Schwieger F, Tebbe CC (1998) A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. Appl Environ Microbiol 64:4870–4876
35. Sneath PHA, Sokal RR (1973) Numerical Taxonomy. Freeman, San Francisco
36. Stoffels M, Amann R, Ludwig W, Hekmat D, Schleifer K (1998) Bacterial community dynamics during start-up of a trickle-bed bioreactor degrading aromatic compounds. Appl Environ Microbiol 64:930–939
37. Tchelet R, Meckenstock R, Steinle P, van der Meer JR (1999) Population dynamics of an introduced bacterium degrading chlorinated benzenes in a soil column and in sewage sludge. Biodegradation 10:113–125
38. Tranvik L, Höfle M (1987) Bacterial growth in mixed cultures on dissolved organic carbon from humic and clear waters. Appl Environ Microbiol 53:482–488
39. Utkin I, Dalton DD, Wiegel J (1995) Specificity of reductive dehalogenation of substituted *ortho*-chlorophenols by *Desulfitobacterium dehalogenans* JW/IU-DC1. Appl Environ Microbiol 61:346–351
40. van de Pas BA, Smidt H, Hagen WR, van der Oost J, Schraa G, Stams AJM, de Vos WM (1999) Purification and molecular characterization of *ortho*-chlorophenol reductive dehalogenase, a key enzyme of halorespiration in *Desulfitobacterium dehalogenans*. J Biol Chem 274:20287–20292
41. van der Meer J, Eggen R, Zehnder A, de Vos W (1991) Sequence analysis of the *Pseudomonas* sp. strain P51 tcb gene cluster, which encodes metabolism of chlorinated catechols: evidence for specialization of catechol 1,2-dioxygenases for chlorinated substrates. J Bacteriol 173:2425–2434
42. van der Meer J, Roelofson W, Schraa G, Zehnder A (1987) Degradation of low concentrations of dichlorobenzenes and 1,2,4-trichlorobenzene by *Pseudomonas* sp. strain P51 in nonsterile soil columns. FEMS Microbiol Ecol 45:333–341
43. van der Meer J, Werklen C, Nishino S, Spain J (1998) Evolution of a pathway for chlorobenzene metabolism leads to natural attenuation in contaminated groundwater. Appl Environ Microbiol 64:4185–4193
44. Van Limbergen H, Topp EM, Verstraete W (1998) Bioaugmentation in activated sludge: current features and future perspective. Appl Microbiol Biotechnol 50:16–23
45. Watanabe K, Yamamoto S, Hino S, Harayama S (1998) Population dynamics of phenol-degrading bacteria in activated sludge determined by gyrB-targeted quantitative PCR. Appl Environ Microbiol 64:1203–1209
46. Weinbauer MG, Beckmann C, Höfle MG (1998) Utility of green fluorescent nucleic acid dyes and aluminum oxide membrane filters for rapid epifluorescence enumeration of soil and sediment bacteria. Appl Environ Microbiol 64:5000–5003
47. Weiß H, Kopinke F, Popp P, Wünsche L (1998) In situ remediation research in a complexly contaminated aquifer: the SAFIRA test site at Bitterfeld. Germany-NATO/CCMS Pilot Study Special Session 229:84–91
48. Wikstrom P, Hagglund L, Forsman M (2000) Structure of a natural microbial community in a nitroaromatic contaminated groundwater is altered during biodegradation of extrinsic, but not intrinsic substrates. Microb Ecol 39:203–210
49. Zylstra G, McCombie W, Gibson D, Finette B (1988) Toluene degradation by *Pseudomonas putida* F1: genetic organization of the tod operon. Appl Environ Microbiol 54:1498–1503