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BIODEGRADATION AND BIOREMEDIATION OF CHLORINATED HYDROCARBONS IN SOIL AND GROUNDWATER

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Introduction

Until the middle of 20th century all the pollutants discharged into the environments were considered to be readily degraded by microorganisms and their degradation products recycled. However, as various industries developed, the pollutants have been increasingly diversified in species, persistency and toxicity, and hence their treatments have required more advanced technologies. This indicates that only use of the conventional physical and chemical treatment technologies cannot guarantee the ultimate and environment-friendly treatment of complex, toxic and recalcitrant contaminants any more (OECD, Biotechnology for a clean environment, 1994). The environmental biotechnologies (particularly bioremediation), since 1970's, have been frequently covered in the media. Environmental biotechnology appears to be socially and politically acceptable on the basis of environmental protection law in the U.S. In this review, concept and application of biodegradation and bioremediation of some important chlorinated hydrocarbons in soil and groundwater will be discussed.

Sources, Types, Fates, and Toxicity of Hydrocarbons in the Environment

The number, diversity, and complexity of synthetic organic chemicals produced are overwhelming. Organic chemicals are, therefore, ubiquitous and affect every aspect of life on earth. More than 100,000 chemicals have been produced commercially, yet a good information on their environmental fate or impact on human health exists for only quite a limited fraction of them --- perhaps 100 compounds (Englande and Guarino, 1992).

The major sources of the pollutants include petrochemical, plastic, pesticide paint, pulp and metal industries. The pollutants generated from these industries are petroleum products (aliphatic and aromatic components such as light chain hydrocarbons and benzene, toluene, ethylbenzene and xylene; BTEX), styrene, surfactants, chlorinated and non-chlorinated solvent such as methylene chloride, tetrachloromethane, trichloroethylene, CFCs, chlorinated phenolic compounds, and chlorinated benzenes. Most of these chemicals, whether they are derived from petrochemicals or synthetic derivatives of the chemicals, cause toxic effects on organisms including both eukayrotes and prokaryotes. The former organisms can detoxify the chemicals through a biotransformation process and their metabolites are secreted. Otherwise, their biotransformation could generate reactive electrophilic chemical species, leading to cell damage. The prokaryotes, however, can generally degrade and detoxify the chemicals or utilize them unless a critical toxicity effect causes lethality to the cells. Microbial degradation of xenobiotics is crucial in the prediction of the longevity and, thereby, the long-term effect of the toxicant, and may also be crucial in the actual remediation of a contaminated site (Landis and Yu, 1995). In other words, the biodegradation data could provide useful information in terms of risk assessment of the target chemicals to comply legal limits or implementing remediation strategies.

Bioremediation as an Alternative Environmental Remediation Technology in Soil and Groundwater Cleanups

Environmental biotechnology for environmental pollutants can be defined as a use of microorganisms (and/or other organisms) and their processes for socio-economic benefits in environmental protection and restoration (Sayler and Fox, 1991). The technology generally utilizes microorganisms and other living organisms in nature as a vehicle to remove pollutants from the environment, and requires little input of extra energy and resources and is processed under ambient conditions, indicating these systems are believed to be low in cost compared with other clean-up technologies. Bioremediation as one of the environmental biotechnologies, therefore, utilizes living organisms (microbes, plants, algae, etc.) to remediate and restore the polluted environment through biodegradation and stabilization of pollutants. From an industrial perspective, practices of bioremediation require an interdisciplinary research and development in the fields combining life sciences, environmental science and engineering, ecology and geology, etc. On the while, the principal purposes of environmental cleanup programs through bioremediation organized by the Office of Research and Development, EPA is summarized as follows: to make acceptable to the user community and the general public improved and novel biological treatment systems whose aim is the reduction or elimination of the risk associated with hazardous waste and other forms of environmental pollution, and to accomplish this in both an effective and least expensive manner (Lacy, 1991). The fundamental research agenda for environmental biotechnology comprised four elements: microbial strain development and improvement, the development of improved bioanalytical methods for measuring biotechnological processes, and the development and environmental and reactor systems analysis techniques leading to better process understanding, control, and optimization (Sayler and Fox, 1991).

Bioremediation Technologies Used in Cleanup of Chlorinated Pollutants in Soil and Groundwater

In situ Treatment of Chlorinated Hydrocarbons in Groundwater or Aquifers

Accelerated bioremediation: TCE A field study of biodegradation of trichloroethylene (TCE) through methane injection was conducted at the yard of a home in Japan (Eguchi, *et al.*, 2001). Methane, oxygen, nitrate, and phosphate were introduced into groundwater contaminated with 220 g/L of TCE. After a week of biostimulation, methane concentrations gradually decreased below the detection limit. Methane oxidizing bacterial numbers increased from 10 to 104 cells/mL with methane consumptions. During methane injection, 10-20% of TCE removal was observed. The biotransformation yield was 3-13 mgTCE/gCH_4 in this field test. After methane injections were stopped, TCE removal was not observed. These results implied that bioremediation using methane was useful as a safe technology for a TCE-contaminated area near homes.

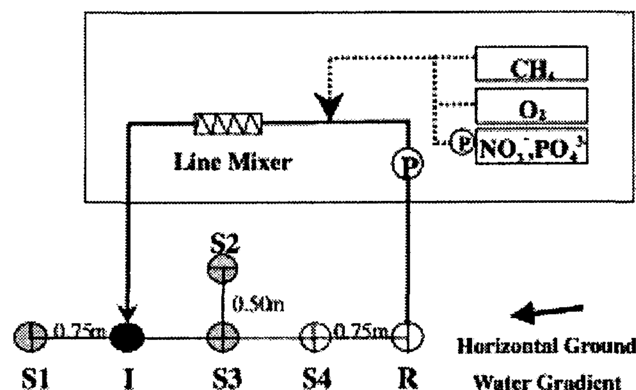


Figure 1. Injection system and placement of the well in the field test.

Natural attenuation (intrinsic bioremediation): Natural attenuation is an *in situ* remediation strategy using natural processes to control or reduce the pollutants. The United States Environmental Protection Agency (USEPA) adopted three tiers of site-specific information as core evidences to document the natural attenuation as following (USEPA, 1999):

- Documented loss of contaminants at the field scale
- Presence and distribution of geochemical and biochemical indicators of natural attenuation
- Direct microbiological evidence

Monitored natural attenuation (MNA) has recently been employed as a feasible groundwater remediation technology in the United States. Area 6 at Dover Air Force Base (Dover, DE) was chosen as a test site to examine the potential for MNA of tetrachloroethene (PCE) and trichloroethene (TCE) in groundwater and aquifer sediments (Michael *et al.*, 2002). Reductive dechlorination likely dominated in the anaerobic portion of the aquifer where PCE and TCE levels were observed to decrease with a simultaneous increase in *cis*-1,2-dichloroethene (*cis*-DCE), vinyl chloride (VC), ethene, and dissolved chloride. Near the anaerobic/aerobic interface, concentrations of *cis*-DCE and VC decreased to below detection limits, presumably due to aerobic biotransformation processes. Therefore, a combination of active anaerobic and aerobic biotransformation processes could cause the natural attenuation to reduce contaminant and daughter product plumes.

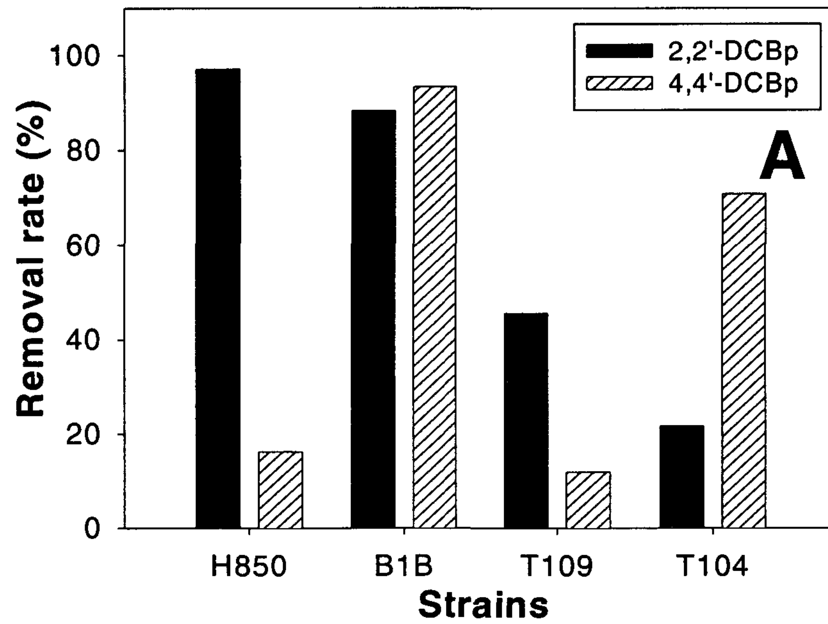
A multi-dimensional and multi-species reactive transport model was developed to facilitate an analysis of natural attenuation design at chlorinated solvent sites (Clement *et al.*, 2000). This model could simulate several simultaneously occurring attenuation processes including aerobic and anaerobic biological degradation processes. The calibrated model reproduced the general groundwater flow patterns, and also, it successfully recreated the observed distribution of tetrachloroethene (PCE), trichloroethene (TCE), dichloro-

ethylene (DCE), vinyl chloride (VC) and chloride plumes. Field-scale decay rates are within the range of values that were previously estimated based on lab-scale microcosm and field-scale transect analyses (Clement *et al.*, 2000).

Compound-specific stable carbon isotope measurements for dissolved PCE and TCE in wells spread throughout the anaerobic portion of the plume confirmed that stable carbon isotope values were isotopically enriched in 13C consistent with the effects of intrinsic biodegradation (Sherwood Lollar *et al.*, 2001). During anaerobic microbial reductive dechlorination of chlorinated hydrocarbons, the light (^{12}C) versus heavy isotope (^{13}C) bonds are preferentially degraded, resulting in isotopic enrichment of the residual contaminant in ^{13}C. Stable carbon isotope signatures were used to quantify the relative extent of biodegradation between zones of the contaminant plume.

Microbial Treatment of PCBs in Soil and Sediments

Microbial treatment of PCBs facilitated by plant natural substrates in soil: A study was performed to examine how plant terpenoids, as natural growth substrates or inducers, would affect the biodegradation of PCB congeners. Various PCB degraders that could grow on biphenyl and several terpenoids were tested for their PCB degradation capabilities. Degradation activities of the PCB congeners, 4,4-dichlorobiphenyl (4,4-DCBp) and 2,2-dichlorobiphenyl (2,2-DCBp), were initially monitored through a resting cell assay technique that could detect their degradation products. The PCB degraders, *Pseudomonas* sp. P166 and *Rhodococcus* sp. T104, were found to grow on both biphenyl and terpenoids ((S)-(-) limonene, *p*-cymene and α -terpinene) whereas *Arthrobacter sp.* B1B could not grow on the terpenoids as a sole carbon source. This indicates that terpenoids, widely distributed in nature, could be utilized as both growth and/or inducer substrate(s) for PCB biodegradation in the environment (Koh *et al.*, 2001).



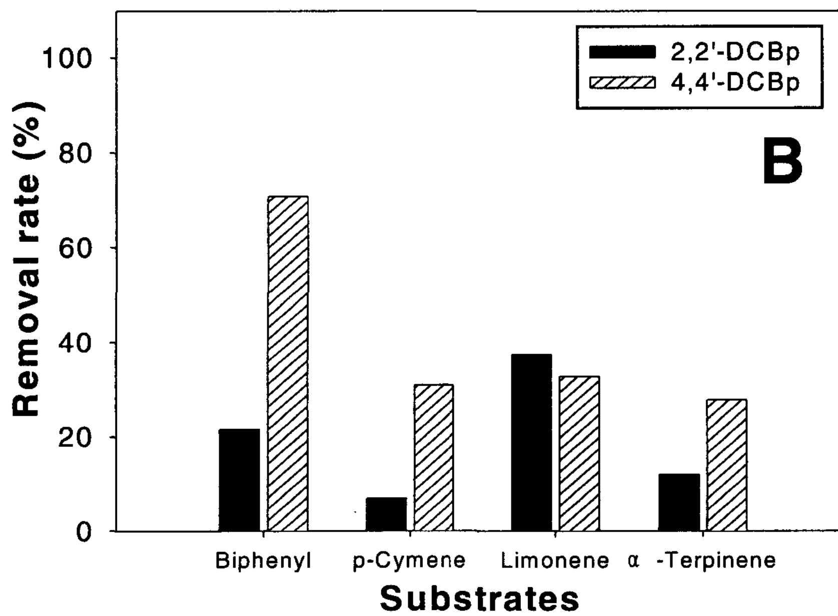


Figure 2. PCB removal rates of *Rhodococcus* sp. T104 grown on biphenyl (A) and various plant terpenoids (B).

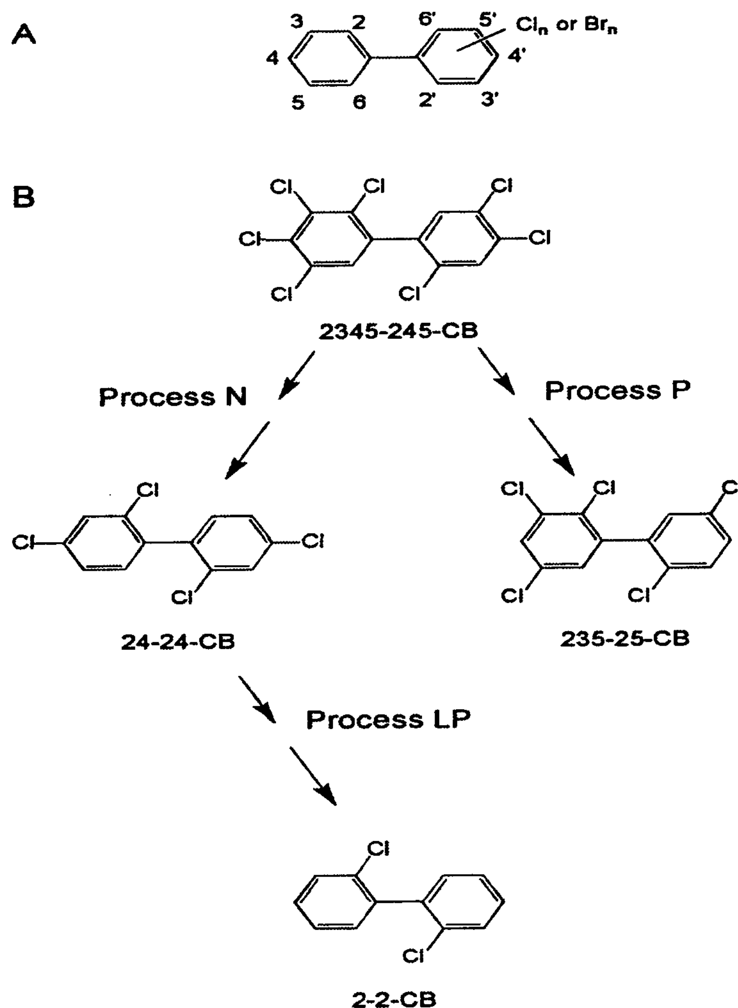


Figure 3. Structure of halogenated biphenyls, showing the numbering scheme (A) and the dechlorination of a major heptachlorobiphenyl by Process N, Processes N plus LP, and Process P (B). Each chlorinated or brominated biphenyl congener can have from 1 to 10 halogens (chlorines or bromines) at the positions shown in panel A. The halogens at positions 2 and 6 on either ring are designated *ortho*, those at carbons 3 and 5 are designated *meta*, and those at carbon 4 are designated *para*. Panel B shows one of the major components of Aroclor 1260 and the terminal dechlorination products produced from this congener by Process N, Processes N plus LP, and Process P. The most extensive chlorine removal results from sequential dechlorination by Processes N and LP.

In Housatonic River and Woods Pond contaminated with Aroclor 1260, certain PCB congeners were found to have the ability to activate or "prime" anaerobic microorganisms in the pond sediment to reductively dehalogenate the Aroclor 1260 residue (Donna *et al.*, 1998)

Congeners containing a *meta* bromine primed Dechlorination Process N (flanked *meta* dechlorination), and congeners containing an unflanked *para* bromine primed Dechlorination Process P (flanked *para* dechlorination). These results imply a major step toward identifying an effective method for accelerating PCB dechlorination *in situ*. It is now necessary to find naturally occurring compounds that are safe and effective primers.

Microbial treatment of PCBs facilitated by natural substrates in marine sediments: Bacterial cultures enriched from Baltimore Harbor (BH) sediments were found to reductively dechlorinate 2,3,5,6-tetrachlorobiphenyl (2,3,5,6-CB) when incubated in a minimal estuarine medium containing short-chain fatty acids under anaerobic conditions with and without the addition of sediment (Cutter *et al.*, 1998). These results indicated that the classical microbial enrichment technique using a minimal medium with a single polychlorinated biphenyl (PCB) congener selected for *ortho* dechlorination of 2,3,5,6-CB. This report is significant in the terms of sustained anaerobic PCB dechlorination in the complete absence of soil or sediment.

Microbial Treatment of Other Organics in Soil and Sediments

The bioremediation of polluted groundwater and toxic waste sites requires bacteria to come into close physical contact with pollutants. Five motile strains of bacteria that use five different pathways to degrade toluene were tested for their ability to detect and swim towards this pollutant (Rebecca *et al.*, 2000). Three of the five strains (*Pseudomonas putida* F1, *Ralstonia pickettii* PKO1, and *Burkholderia cepacia* G4) were attracted to toluene. *Pseudomonas mendocina* KR1 and *P. putida* PaW15 did not show a convincing response.

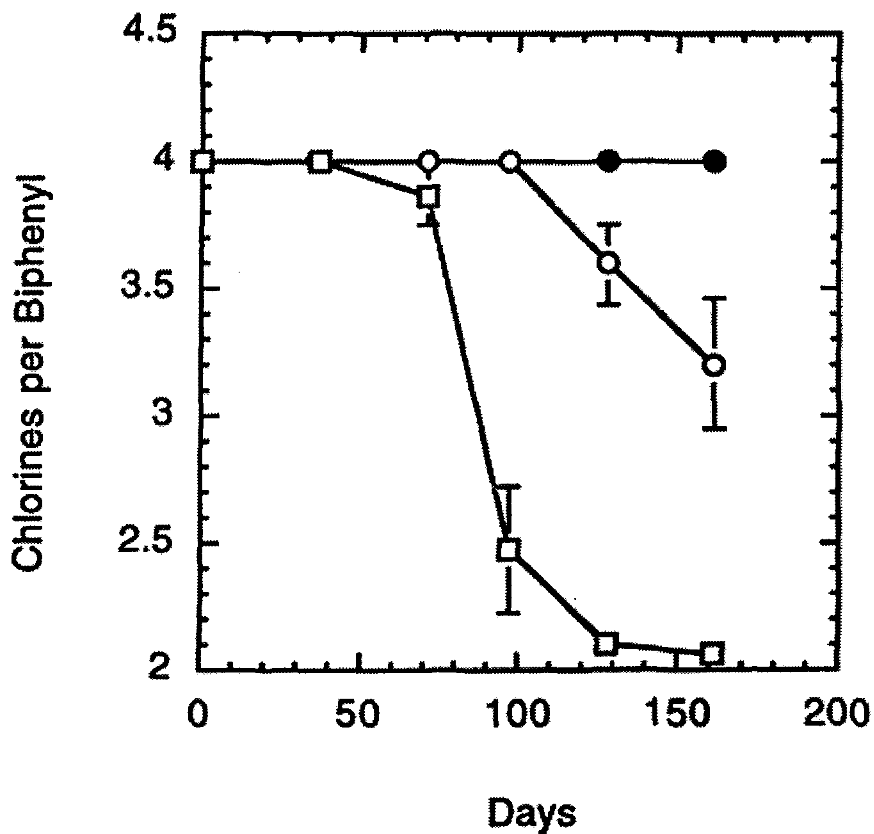


Figure 4. Effect of sediment added to a pre-dechlorination sediment-free culture. The fourth-sequential-transfer sediment-free cultures (pre-dechlorination) were transferred to medium with (□) and without (○) 1.0% sterilized BH sediment. •, killed-cell control. All data are for triplicate cultures. Error bars indicate standard deviations.

This demonstration that soil bacteria were able to sense and swim towards the toxic compounds toluene, benzene, TCE, and related chemicals suggested that the introduction of chemotactic bacteria into selected polluted sites might accelerate bioremediation processes.

Monitoring Technologies for Bioremediation Processes in Soil and Groundwater

Microbial communities within contaminated ecosystems are likely to be dominated by those organisms capable of utilizing and/or surviving toxic contamination. As a result, these communities are typically less diverse than those in non-stressed systems, although the diversity may be influenced by the complexity of chemical mixtures present and the length of time the populations have been exposed. However, when gram-negative bacteria dominate the system (as is usually the case in hydrocarbon-contaminated environments), the insight gained from lipid biomarker analysis primarily concerns nutritional or physiological status with little differentiation among bacterial species. A complementary method by which the shift in such a microbial community structure can be monitored in greater detail is denaturing gradient gel electrophoresis (DGGE). This method takes advantage of the 16S rDNA molecule carried by all bacteria, the sequences of which provide molecular markers for species identification. The method was originally used for profiling microbial populations in environmental samples by Muyzer *et al.* (1993).

In situ Monitoring of the Microbial Communities Involved in Biodegradation of Petroleum Hydrocarbons

The study of microbial diversity and community dynamics is a rapidly growing area in microbial ecology. A rapid advancement in this area has been greatly facilitated by molecular ecological methodologies. Through the use of culture-independent molecular techniques, new insights into the composition of uncultivated microbial communities have been gained. It is now becoming possible to define the causes of time-dependent changes in the health of a stressed ecosystem on the basis of the structural composition of the ecosystem population (Head *et al.*, 1998).

DGGE technique is based on the separation of polymerase chain reaction-amplified fragments of genes coding for 16S rRNA, all the same length, by denaturing gradient gel electrophoresis (DGGE). DGGE analysis of different microbial communities demonstrated the presence of up to 10 distinguishable bands in the separation pattern, which were most likely derived from as many different species constituting these populations, and thereby generated a DGGE profile of the populations.

PLFA analysis indicated that by week 14 the microbial community structures of the oiled plots were becoming similar to those of the unoiled controls from the same time point, but DGGE analysis suggested that major differences in the bacterial communities remained.

A study was undertaken to gain insight on the progress of natural attenuation and enhanced bioremediation during a controlled oil spill field experiment in Delaware (Venosa *et al.*, 1996). The results of PLFA analysis demonstrated a community shift in all plots from primarily eukaryotic biomass to gram-negative bacterial biomass with time. PLFA profiles from the oiled plots suggested increased gram-negative biomass and adaptation to metabolic stress compared to unoiled controls. PCR-DGGE analysis of untreated control plots showed a simple, dynamic dominant population structure throughout the experiment. This DGGE pattern disappeared in all oiled plots, reflecting that the structure and diversity of the dominant bacterial community changed substantially. No consistent differences were detected between nutrient-amended and indigenous inoculum-treated plots, but both differed from the oil-only plots. Representative bands were excised for sequence analysis and indicated that oil treatment encouraged the growth of gram-negative microorganisms within the *alpha-proteobacteria* and *Flexibacter- Cytophaga-Bacteroides* phylum (FIG. 5) (MacNaughton *et al.*, 1999).

Petroleum-contaminated groundwater discharged from underground crude oil storage cavities (cavity groundwater) harbored more than 10^6 microorganisms ml^{-1}, a density 100 times higher than the densities in groundwater around the cavities (control groundwater).

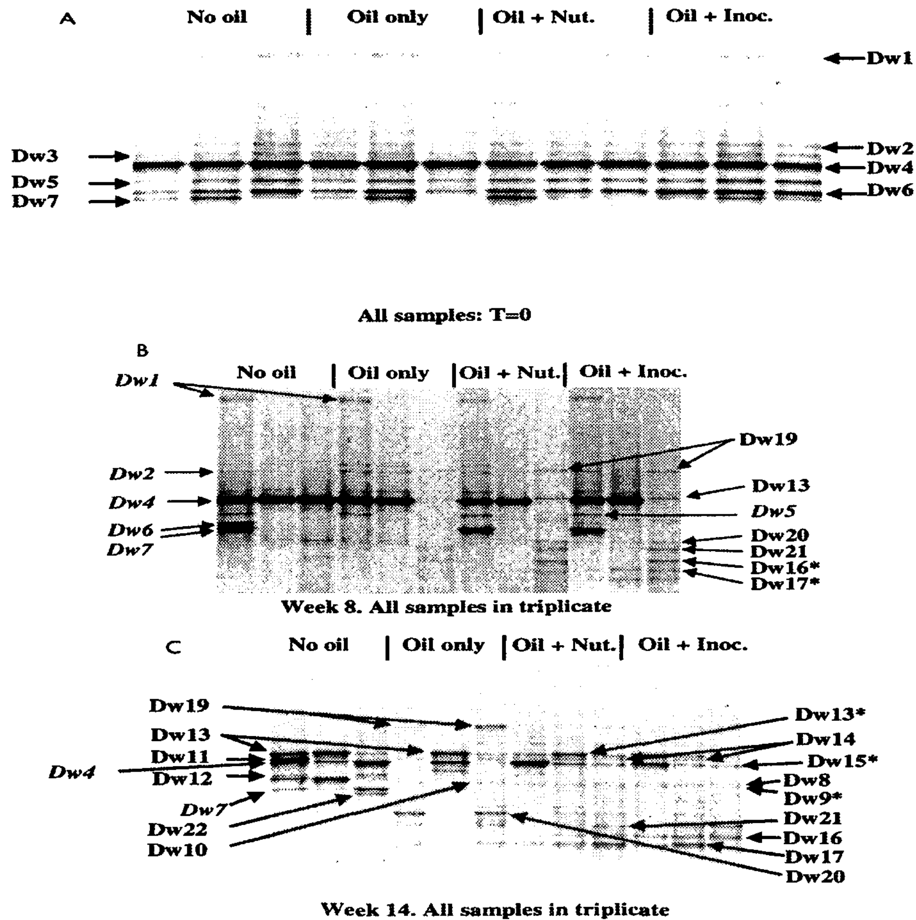


Figure 5. DGGE analysis of bacterial communities at three time points. Amplified products were separated on a gradient of 15 to 55% denaturant. The region shown represents approximately 20 to 45% denaturant, in which all visible bands formed. All labeled bands were excised from the gel, reamplified, and subjected to sequence analysis. Bands marked with an asterisk failed to generate legible sequences by direct analysis. (A) Community structures at time zero. Time zero was defined as 4 days after oil was added to the plots and was the time point at which accelerated remediation techniques were initiated (amendment with nutrients [Nut.] or nutrients plus inoculum [Inoc.]). (B) Community structures after 8 weeks of treatment. Most, but not all, oiled plots had developed complex banding patterns compared to the unoiled control samples, indicating an even distribution of numerous dominant species. (C) Community structures after 14 weeks of treatment. The banding patterns of all oiled plots were complex compared to unoiled plots. Two bands (Dw8 and Dw9) were visible only in oiled plots that had also received nutrient amendment

To characterize bacterial populations growing in the cavity groundwater, 46 PCR-amplified almost full-length 16S ribosomal DNA (rDNA) fragments were cloned and sequenced, and 28 different sequences were obtained. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified partial 16S rDNA fragments showed that one band was detected most strongly in cavity groundwater profiles independent of storage oil type and season. The sequence of this major band was identical to the sequences of most of the cluster 1 clones. Fluorescence in situ hybridization (FISH) indicated that the cluster 1 population accounted for 12 to 24% of the total bacterial population. These results imply that the novel members of the epsilon subclass of the *Proteobacteria* grow as major populations in the petroleum-contaminated cavity groundwater (Figure 6) (TABLE 1) (Watanabe *et al.*, 2000).

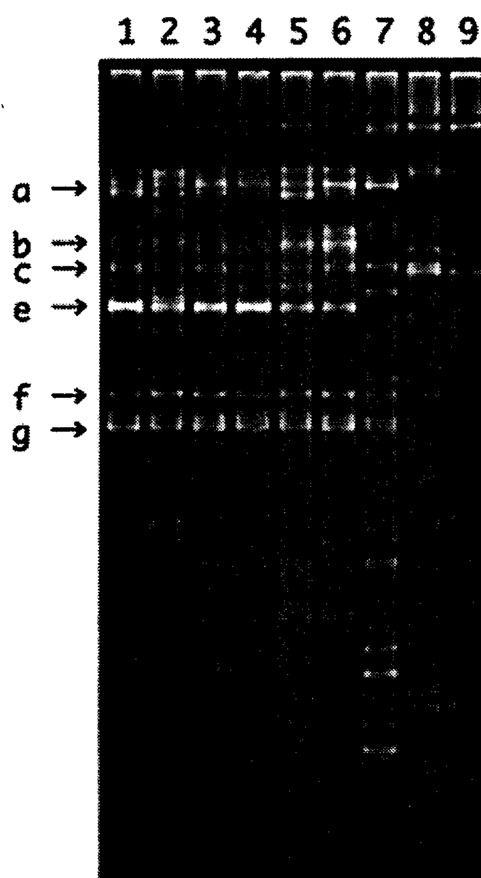


Figure 6. DGGE profiles of the partial 16S rDNA fragments, showing similarities and differences in the bacterial population structures in groundwater samples. Lane 1, TK101 cavity groundwater obtained in May 1998; lane 2, TK101 cavity groundwater obtained in July 1998; lane 3, TK101 cavity groundwater obtained in November 1998; lane 4, TK101 cavity groundwater obtained in March 1999; lane 5, TK102 cavity groundwater obtained in July 1998; lane 6, TK103 cavity groundwater obtained in July 1998, lane 7, injected water obtained in July 1998; lane 8, control groundwater 1 obtained in July 1998; lane 9, control groundwater 2 obtained in July 1998.

Table 1. Sequence analysis of major DGGE bands.

DGGE band[a]	Length (bases)	Database search: Phylogenetically related organism (accession no.)	Database search: % Identity	Identical clone(s)
a	189	Flavobacterium ferrugineum (M62798)	93	
b	169	Arcobacter nitrofigilis (L14627)	100	1006
c	173	Meiothermus ruber (Y13594)	83	
d	194	Pseudomonas sp. (D37925)	100	
e	169	Thiomicrospira denitrificans (L40808)	97	1005, 1008, 1011, 1028, 1043, 1047, 1051, 1054, 1061
f	194	Hydrogenophaga taeniospiralis (AF078768)	90	
g	194	Burkholderia phenazinium (U96936)	95	

[a] DGGE bands marked in Fig. 5 were sequenced.

SRB were found to grow on environmental contaminants such as petroleum hydrocarbon (PHC) constituents (e.g., benzene, toluene, ethylbenzene, xylenes, naphthalene, phenanthrene, and alkanes) and halogenated compounds (Ensley *et al.*, 1995; Zhang *et al.*, 1997). A survey of 38 PHC-contaminated aquifers revealed that on average, SO_4^{2-} reduction was responsible for 70% of PHC attenuation (Wiedemeier *et al.*, 1999).

In situ Monitoring of the Microbial Communities Involved in Biodegradation of Chlorinated Hydrocarbons

A microorganism whose growth is linked to the dechlorination of polychlorinated biphenyls (PCBs) with doubly flanked chlorines was identified (Qingzhong, et al., 2002). Here identification was made by reductive analysis of community 16S ribosomal DNA (rDNA) sequences from a culture enriched in the presence of 2,3,4,5-tetrachlorobiphenyl (2,3,4,5-CB), which was dechlorinated at the *para* position. Denaturing gradient gel electrophoresis (DGGE) analysis of total 16S rDNA extracted from the culture showed identification of three operational taxonomic units (OTUs 1, 2, and 3). OTU 1 was always detected when 2,3,4,5-CB or other congeners with doubly flanked chlorines were present and dechlorinated. Only OTUs 2 and 3 were detected in the absence of PCBs and when other PCBs (i.e., PCBs lacking doubly flanked chlorines) were not dechlorinated. These results indicated that OTU 1 represents the dechlorinating bacterium growing in a coculture with a *Desulfovibrio* sp. The 16S rDNA sequence of OTU 1 is most similar to the 16S rDNA sequence of bacterium o-17 (89% similarity), an *ortho*-PCB-dechlorinating bacterium. Therefore, the dechlorinator appeared to reductively dechlorinate congeners with doubly flanked chlorines when it is supplied with formate or H_2-CO_2 (80:20).

In situ Monitoring of the Microbial Activities Involved in Biodegradation of Petroleum and Chlorinated Hydrocarbons

Procedures were developed for isolating and characterizing in *situ*-transcribed mRNA from groundwater microorganisms catabolizing naphthalene at a coal tar waste-contaminated site (Wilson *et al.*, 1995). Here RNA was extracted from the frozen filters by boiling sodium dodecyl sulfate lysis and acidic phenol-chloroform extraction. Transcript characterization was performed with a series of PCR primers designed to amplify *nahAc* homologs. Several primer pairs were found to amplify nahAc homologs representing the entire diversity of the naphthalene-degrading genes. The environmental RNA extract was reverse transcribed, and the resultant mixture of cDNAs was amplified by PCR. A digoxigenin-labeled probe mixture hybridized under stringent conditions with the corresponding PCR products from naphthalene-degrading bacteria carrying a variety of *nahAc* homologs, indicating that diverse dioxygenase transcripts had been retrieved from groundwater. Sequence comparisons revealed two major groups related to the dioxygenase genes *ndoB* and *dntAc,* previously cloned from *Pseudomonas putida* NCIB 9816-4 and *Burkholderia* sp. strain DNT, respectively. A distinctive subgroup of sequences was found only in experiments performed with the undiluted cDNA preparation. The retrieved sequences represented greater diversity than had been detected at the study site by culture-based approaches.

Biosensor Technology for Monitoring Bioremediation Processes

The application of biosensors is favorable due to some generally claimed advantages: intrinsic specificity, low costs, fast analyses, and minimal requirements for sample pretreatment. However, widespread application of biosensors is still considerably hampered by the low stability of biosensors and insufficient detection limits. Enzyme-based amperometric biosensors based on tyrosinase, laccase, or peroxidase have been previously reported.

A green fluorescent protein-based Pseudomonas fluorescens strain A506 biosensor was constructed and characterized for its potential to measure benzene, toluene, ethylbenzene, and related compounds in aqueous solutions (Stiner *et al.*, 2002). The biosensor is based on a plasmid carrying the toluene-benzene utilization (tbu) pathway transcriptional activator TbuT from *Ralstonia pickettii* PKO1 and a transcriptional fusion of its promoter PtbuA1 with a promoterless *gfp* gene on a broad-host-range promoter probe vector. The biosensor cells were readily induced, and fluorescence emission after induction periods of 3 h correlated well with toluene, benzene, ethylbenzene, and trichloroethylene concentrations. Our experiments using flow cytometry show that intermediate levels of gfp expression in response to toluene reflect uniform induction of cells. As the toluene concentration increases, the level of gfp expression per cell increases until saturation kinetics of the TbuT-PtbuA1 system are observed. Each inducer had a unique minimum concentration that was necessary for induction, with K_{app} values that ranged from 3.3 ± 1.8 μM for toluene to 35.6 ± 16.6 μM for trichloroethylene (means ± standard errors of the means), and maximal fluorescence response. These results demonstrated the potential for green fluorescent protein-based bacterial biosensors to measure environmental contaminants.

A bacterial biosensor for benzene, toluene, and similar compounds has been constructed, characterized, and field tested on contaminated water and soil. The biosensor is based on a plasmid incorporating the transcriptional activator xylR from the TOL plasmid of Pseudomonas putida mt-2. The biosensor cells were also shown to detect BETX (benzene, toluene, and xylene) contamination in soil samples. These results demonstrate the capability of such a bacterial biosensor to accurately measure environmental contaminants and suggest a potential for its inexpensive application in field-ready assays (Willardson *et al.*, 1998). A tod-luxCDABE fusion was constructed and introduced into the chromosome of Pseudomonas putida F1, yielding the strain TVA8. This strain was used to examine the induction of the tod operon when exposed to benzene, toluene, ethylbenzene, and xylene (BTEX) compounds and aqueous solutions

of JP-4 jet fuel constituents. There was an increasing response to toluene concentrations from 30 μ g/liter to 50 mg/liter, which began to saturate at higher concentrations. The detection limit was 30 μ g/liter. The transposon insertion was stable and had no negative effect on cell growth (Applegate *et al.*, 1998).

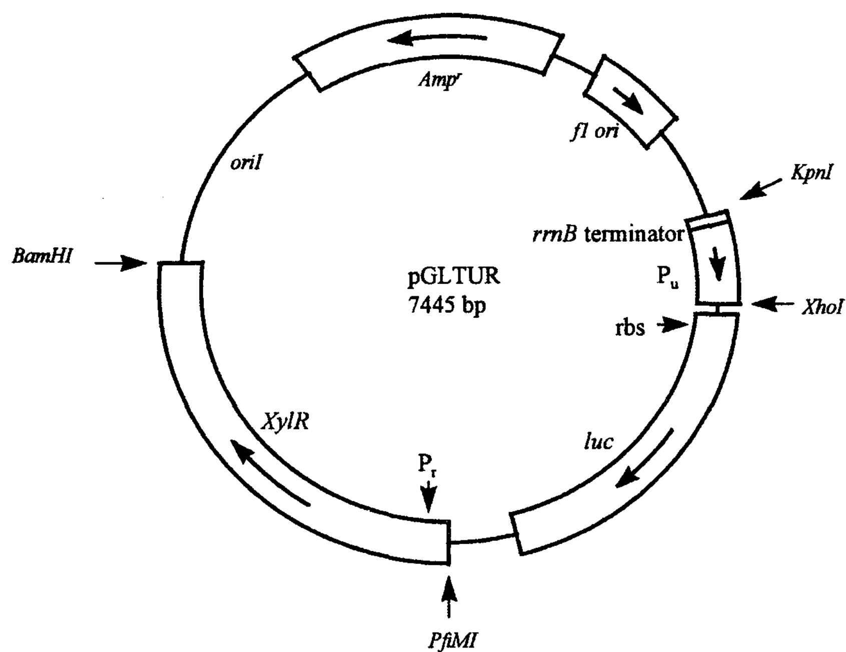


FIG. 7. Plasmid map of the pGLTUR biosensor construct. Important features of the toluene biosensor are indicated, including the location and orientation of the P_u promoter, the *E. coli rrnB* transcription terminator sequence, the *luc* luciferase gene, the P_r promoter, and the *xylR* transcriptional activator gene. Restriction sites used to insert P_u and *xylR* are shown for reference. rbs, ribosome binding site.

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