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Functional genes and gene array analysis as tools for monitoring hydrocarbon biodegradation

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Faculty of Biosciences
Department of Biological and Environmental Sciences
University of Helsinki

ACADEMIC DISSERTATION

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tel. växel 020 722 111, fax 020 722 7001

VTT Technical Research Centre of Finland, Vuorimiehentie 3, P.O. Box 1000, FI-02044 VTT, Finland
phone internat. +358 20 722 111, fax + 358 20 722 7001

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Keywords aliphatic hydrocarbon, aromatic hydrocarbon, biodegradation, bioremediation, BTEX, functional gene, gene array, PAH, process monitoring, real-time PCR

Abstract

Bioremediation, which is the exploitation of the intrinsic ability of environmental microbes to degrade and remove harmful compounds from nature, is considered to be an environmentally sustainable and cost-effective means for environmental clean-up. However, the successful implementation of bioremediation is dependent on several factors, such as the characteristics and the amount of the pollutant, environmental conditions and the degradation capacity and activity of the microbial communities. As a result, a comprehensive understanding of the biodegradation potential of the microbial communities and their response to decontamination measures is required for the effective management of bioremediation processes.

In this thesis, the potential to use functional genes encoding hydrocarbon-degradative enzymes as indicators of aerobic hydrocarbon biodegradation was investigated. Small-scale, functional gene macro- and microarrays targeting aliphatic, monocyclic aromatic hydrocarbon and low molecular weight polycyclic aromatic hydrocarbon (PAH) biodegradation were developed in order to monitor the biodegradation of mixtures of hydrocarbons. Several genes from each biodegradation pathway were attached to the arrays in order to allow the detection of biodegradation pathways. The validity of the array analysis in monitoring hydrocarbon biodegradation was evaluated in microcosm studies and field-scale bioremediation processes by comparing the hybridization signal intensities to hydrocarbon mineralization, real-time polymerase chain reaction (PCR), dot blot hybridization and both chemical and microbiological monitoring data.

The amount of functional genes, quantified by real-time PCR and dot blot hybridization was in good agreement with hydrocarbon biodegradation. In a PAH-contaminated soil slurry microcosm, the quantification of naphthalene

dioxygenase gene copies enabled monitoring of the growth of intrinsic or inoculated naphthalene-degrading bacteria during naphthalene biodegradation. As a result, better understanding of the differences in mineralization efficiencies that were observed between treatments was obtained.

The hybridization signals obtained from the functional gene array analysis were also in good agreement with the mineralization of specific hydrocarbons. Biodegradation of several hydrocarbons could be monitored simultaneously, as demonstrated by toluene and naphthalene biodegradation in a mixed bacterial culture.

In the field-scale bioremediation processes, the combination of conventional physico-chemical and microbiological monitoring methods with the detection and enumeration of hydrocarbon-degradative genes provided important additional information for process optimization and design. In creosote-contaminated groundwater, the increased abundance of PAH-degradative genes detected by the functional gene microarray demonstrated that the aerobic PAH-biodegradation potential that was present at the site, but restrained under the oxygen-limited conditions, could be successfully stimulated with air sparging and nutrient infiltration. During *ex situ* bioremediation of diesel oil- and lubrication oil-contaminated soil, the changes in the abundance of alkane-biodegradative genes observed by functional gene array early in the process revealed inefficient hydrocarbon biodegradation, caused by poor aeration during composting. The array analysis specifically detected both upper and lower biodegradation pathway genes that are required for degradation of hydrocarbons. Genes involved in the degradation of different hydrocarbons were also discriminated. Hydrocarbon-degrading bacteria representing 1 % of the microbial community could be detected without prior PCR amplification.

Molecular biological monitoring methods based on functional genes provide powerful tools for increasing our understanding of the effects of remediation treatments on the contaminant-degrading microbial communities. As a result, more efficient remediation processes can be developed. With careful assay design, the analysis can be directed to relevant biodegradation functions rather than specific genotypes. The parallel detection of several functional genes by gene array analysis is an especially promising tool for monitoring the biodegradation of mixtures of hydrocarbons.

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Tiivistelmä

Biopuhdistuksessa pilaantunutta ympäristöä kunnostetaan haitallisia yhdisteitä hajottavia, ympäristössä luonnollisesti esiintyviä mikrobeja hyväksi käyttäen. Sitä pidetään ympäristön kannalta kestävänä sekä taloudellisesti kannattavana kunnostusmenetelmänä. Biopuhdistuksen tehokkuus riippuu kuitenkin useista tekijöistä, joita ovat muun muassa hajotettavan yhdisteen ominaisuudet ja määrä, ympäristöolosuhteet sekä mikrobiyhteisöjen biohajotuspotentiaali ja aktiivisuus. Biologisten puhdistusprosessien hallitseminen vaatiikin ymmärrystä mikrobiyhteisöjen hajotuspotentiaalista sekä yhteisöissä kunnostuksen seurauksena tapahtuvista muutoksista.

Tässä väitöskirjatutkimuksessa tutkittiin hiilivetyjen hajotuksesta vastaavia entsyymejä koodaavien toiminnallisten geenien soveltuvuutta hiilivetyjen aerobisen biohajoamisen seurantaan. Jotta useiden hiilivetyjen biohajoamista voitaisiin tutkia samanaikaisesti, tutkimuksessa kehitettiin suoraketjuisten sekä monoaromaattisten hiilivetyjen ja pienimolekyylisten polyaromaattisten hiilivetyjen (PAH) hajotusgeeneistä koostuvat pienimuotoiset makro- ja mikroarrayt. Jokaiselta hajotusreitiltä valittiin useita geenejä hajotusreittien tunnistamiseksi. Arraymenetelmien toimivuutta hiilivetyjen biohajoamisen seurannassa arvioitiin sekä laboratoriomittakaavan mikrokosmoskokeissa että kenttämittakaavan biopuhdistusprosesseissa vertaamalla arraytuloksia hiilivetyjen mineralisaatioon, reaaliaikaiseen polymeraasiketjureaktioon (PCR), dot blot -hybridisaatioon sekä kemiallisilla ja mikrobiologisilla seurantamenetelmillä saatuihin tuloksiin.

Reaaliaikaisella PCR:llä ja dot blot -hybridisaatiolla mitattu toiminnallisten geenien määrä oli hyvässä yhteydessä hiilivetyjen mineralisaatioon. PAH:lla pilaantuneessa maasuspensiossa naftaleenidioksigenaasi-geenin kopioiden määrän avulla oli mahdollista seurata luontaisten tai näytteisiin lisättyjen naftaleenia hajottavien bakteerien kasvua naftaleenin biohajoamisen aikana ja siten ymmärtää käsittelyjen välillä havaittuja mineralisaatiotehokkuuksien eroja.

Myös geeniarraylla saadut tulokset olivat hyvässä yhteydessä hiilivetyjen hajoamiseen. Kuten tolueenia ja naftaleenia hajottavien *Pseudomonas putida* -kantojen sekaviljelmä osoitti, useiden hiilivetyjen biohajoamista voitiin seurata samanaikaisesti.

Hajotusgeenien tutkimisen yhdistäminen perinteisiin fysikaaliskemiallisiin ja mikrobiologisiin seurantamenetelmiin lisäsi prosessien optimoinnissa ja suunnittelussa tarvittavaa tietoa myös kenttämittakaavan puhdistusprosesseissa. Kreosootilla pilaantuneessa pohjavedessä geeniarraylla havaittu PAH:n hajotukseen osallistuvien geenien kohonnut määrä osoitti, että pilot-mittakaavan reaktiivisella seinämällä voitiin aktivoida kohteessa esiintyvää, mutta hapettomien olosuhteiden rajoittamaa, hajotuspotentiaalia. Diesel- ja voiteluöljyllä pilaantuneen maan *ex situ* -kunnostuksessa suoraketjuisten hiilivetyjen hajotusgeenien määrässä funktionaalisella geeniarraylla havaitut muutokset paljastivat jo prosessin alkuvaiheessa, että biohajoamisen tehokkuus heikentyi puutteellisen ilmastuksen vuoksi. Arrayanalyysi tunnisti spesifisesti hajotusgeenejä sekä ylemmältä että alemmalta hiilivetyjen hajotukseen tarvittavalta hajotusreitiltä. Myös erilaisten hiilivetyjen hajotukseen osallistuvat geenit voitiin tunnistaa spesifisesti. Hiilivetyjä hajottavat bakteerit oli mahdollista tunnistaa ilman hajotusgeenien PCR-monistusta, kun ne edustivat yhtä prosenttia koko mikrobiyhteisöstä.

Toiminnallisiin geeneihin perustuvat molekyylibiologiset seurantamenetelmät muodostavat tehokkaita työkaluja, joiden avulla opimme paremmin ymmärtämään kunnostuskäsittelyjen vaikusta mikrobiyhteisöihin ja siten kehittämään entistä tehokkaampia kunnostusprosesseja. Oikealla suunnittelulla analyysi voidaan kohdentaa yksittäisen genotyyppien sijaan kunnostuksen kannalta keskeisiin hajotusaktiivisuuksiin. Useiden hajotusgeenien samanaikainen tunnistaminen geeniarrayhybridisaatioon perustuen on erityisen lupaava menetelmä useista hiilivedyistä koostuvien seosten biohajoamisen seurannassa.

Preface

This thesis work was carried out at the Technical Research Centre of Finland (VTT) during 2003–2008. Part of the work was performed in the VTT's Clean World Research Program. I thank Professor Juha Ahvenainen (2002–2007) and Dr. Anu Kaukovirta-Norja (2007–2008), the directors of VTT Biotechnology, for providing the excellent working facilities.

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Lahti, May 2009

Mari Nyyssönen

Contents

Appendices of this publication are not included in the PDF version. Please order the printed version to get the complete publication (http://www.vtt.fi/publications/index.jsp).

List of publications

This thesis is based on the following publications, which are referred in the text by the Roman numerals (I–IV).

I Nyyssönen, M., Piskonen, R. and Itävaara, M. 2006. A targeted real-time PCR assay for studying naphthalene degradation in the environment. Microbial Ecology, Vol. 52, pp. 533–543.

II Piskonen, R., Nyyssönen, M., and Itävaara, M. 2008. Evaluating the biodegradation of aromatic hydrocarbons by monitoring of several functional genes. Biodegradation, Vol. 19, pp. 883–895.

III Nyyssönen, M., Piskonen, R. and Itävaara, M. 2008. Monitoring aromatic hydrocarbon biodegradation by functional marker genes. Environmental Pollution, Vol. 154, pp. 192–202.

IV Nyyssönen, M., Kapanen, A., Piskonen, R., Lukkari, T. and Itävaara, M. 2009. Functional genes reveal the intrinsic PAH biodegradation potential in creosote-contaminated groundwater following in situ biostimulation. Applied Microbiology and Biotechnology, Vol. 84, pp. 169–182.

Unpublished data will also be presented.

The author’s contribution

I Mari Nyyssönen planned the experiment and performed the experimental work. She interpreted the results, wrote the paper and is the corresponding author. The work was supervised by Reetta Piskonen and Merja Itävaara.

II Reetta Piskonen planned the experiment and is the corresponding author. Mari Nyyssönen performed the experimental work. Mari Nyyssönen and Reetta Piskonen interpreted the results and wrote the paper. Merja Itävaara supervised the work and assisted in writing of the paper.

III Mari Nyyssönen planned the experiment and interpreted the data together with Reetta Piskonen. Mari Nyyssönen conducted the experimental work. She wrote the paper and is the corresponding author. Reetta Piskonen and Merja Itävaara supervised the work and assisted in writing and revision of the paper.

IV Mari Nyyssönen planned the experiment and performed the experimental work. Tuomas Lukkari provided the samples and field data. Mari Nyyssönen interpreted the results with Anu Kapanen and Reetta Piskonen. Mari Nyyssönen wrote the paper and is the corresponding author. Merja Itävaara assisted in writing and revision of the paper.

List of symbols

BTEX	Benzene, toluene, ethylbenzene and xylenes
DAPI	4’, 6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
FDA	Fluorescein diacetate
FID	Flame ionization detector
HMW	High molecular weight (PAHs containing >3 rings)
LMW	Low molecular weight (PAHs containing 2–3 rings)
PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
TSA	Tyramidine signal amplification
GC	Gas chromatography
MS	Mass spectrometry
WGA	Whole genome amplification

1. Introduction

Aliphatic and aromatic hydrocarbons are wide-spread and natural components in the environment. They are formed naturally in geochemical processes from buried biomass as well as in microbial and plant metabolism (Jüttner and Henatsch 1986, Kolattukudy 1968, Tissot and Welte 1984).

Many hydrocarbons have important functions in the environment and their biological transformation to carbon dioxide, biomass and energy forms part of the natural carbon cycle. However, due to human activities, such as the burning of organic matter and fossil fuels, the increased industrial activity and the discharge of oil and petroleum products, the amount of hydrocarbons in the environment has increased considerably.

Aliphatic and aromatic hydrocarbons have detrimental effects on environmental quality and health. Many are considered toxic, mutagenic and carcinogenic and they may accumulate in food chains (Nikunen et al. 2000). The release of aliphatic and aromatic hydrocarbons into the environment can also cause physically and aesthetically adverse effects, such as film formation on shorelines or smell. Altogether, these effects result in land degradation and water pollution limiting land use and damaging the ecosystems which all forms of life are dependent on (Peterson et al. 2003).

Environmental contamination and decontamination are regulated by environmental laws and regulations. In Finland, the Environment Protection Act regulates the prevention of pollution, the remediation of contaminated environments and the responsibilities related to both environmental contamination and clean-up (86/2000). In order to facilitate and guide decision making, evaluation of the extent of soil contamination and the need for environmental clean-up, risk assessment-based soil guideline values and soil criteria have been assigned for 52 compounds, including both aliphatic (<300 mg kg^{-1} soil) and aromatic hydrocarbons (BTEX and EPA16 PAHs <1 and 15 mg kg^{-1} soil,

respectively) (214/2007). In the case of groundwater pollution and remediation, the maximum concentration values for water used for household consumption and World Health Organization's guidelines for drinking water quality are used as guidelines (461/2001, WHO 2004).

Bioremediation is considered as an environmentally and economically attractive alternative to physico-chemical environmental decontamination methods, such as land filling, containment and incineration. In the optimal case, bioremediation using the intrinsic ability of environmental microorganisms to transform environmental contaminants into less harmful products, results in complete degradation and removal of contaminants from polluted environments while, at the same time, preserving and/or restoring the biological value of the environment.

However, several criteria must be fulfilled in order to successfully implement bioremediation. The contaminants that need to be degraded must be biodegradable and bioavailable. Microorganisms capable of contaminant biodegradation and reduction of contaminant concentrations to safe levels must be present at the site. If the site does not harbour an intrinsic biodegradation potential, it must be bioaugmented with contaminant-degrading microbes. Environmental conditions must be appropriate to sustain microbial growth and active biodegradation. Toxic products must not be produced during biodegradation (Andreoni and Gianfreda 2007).

A bioremediation program consists of four steps: i) investigation of the site and the extent of contamination, ii) design and development of a treatment method, iii) implementation of bioremediation measures, and iv) monitoring of the effectiveness of the bioremediation (Sarkkila et al. 2004). A good understanding of the presence and activity of contaminant-degrading microorganisms is required in all stages.

1.1 Characteristics of n-alkanes, BTEX and PAHs

Aliphatic and aromatic hydrocarbons are reduced compounds that lack functional groups and have very low chemical reactivity (Figure 1). The aliphatic n-alkanes are major components of crude oil and natural gas (Tissot and Welte 1984). Alkanes are highly hydrophobic and, depending on their molecular weight, exist as either gases (C_1–C_4), liquids (C_5–C_{17}) or solids (C_{18}–C_{38}) at physiological temperatures. They also have very low solubility in water

(5.2×10^{-5} mg l^{-1} for hexadecane) and extremely low chemical reactivity, which limits their biodegradation in the environment (Watkinson and Morgan 1990).

The unsubstituted or alkyl-substituted monocyclic aromatic hydrocarbons, benzene, toluene, etylbenzene and isomers of xylene (BTEX), are constituents of petroleum products and industrial solvents (Figure 1). BTEX are acutely toxic and have a carcinogenic effect under long-term exposure. Because they are volatile and water soluble, BTEX are mobile in the environment and, therefore, of great toxicological concern as groundwater contaminants (Nikunen et al. 2000).

Polycyclic aromatic hydrocarbons (PAHs) are a group of more than one hundred compounds that consist of two or more fused benzene rings in a linear, angular or clustered arrangement (Figure 1). In the environment, PAHs usually exist in complex mixtures rather than as single compounds. They are released into the environment during the combustion of fossile fuels and in traffic exhaust, as well as from petroleum and coal tar spills. PAHs are toxic, and the high molecular weight (HMW) PAHs also mutagenic and carcinogenic (Nikunen et al. 2000). Similar to the n-alkanes, the hydrophobicity of PAHs increases with an increase in molecular weight and angularity: While the water solubility of the two-ring naphthalene is 31.7 mg l^{-1}, it is only 0.003 mg l^{-1} for benzo[a]pyrene which contains five aromatic rings (Figure 1) (Nikunen et al. 2000). As a result, the HMW PAHs are very persistent in the environment.

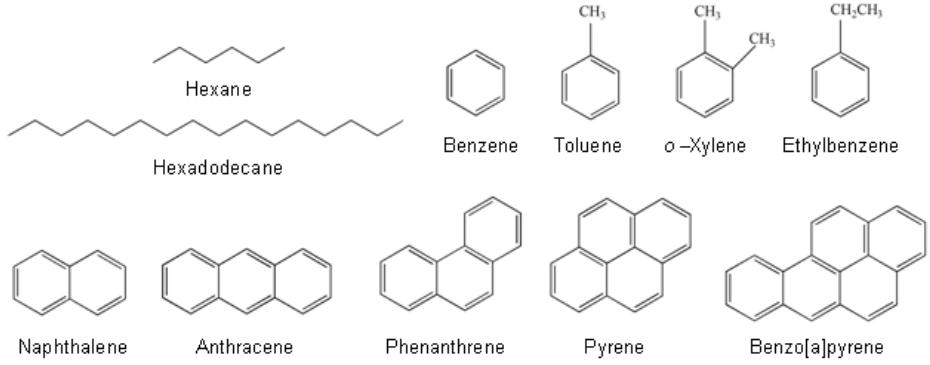

Figure 1. Chemical structure of some aliphatic and aromatic hydrocarbons.

1.2 Hydrocarbon biodegradation

In the environment, the aliphatic and aromatic hydrocarbons are subjected to two processes, transportation or degradation, depending on the environmental conditions, characteristics of the compound and properties of the environmental matrix. Volatilization, adsorption and desorption on soil surfaces, deposition and other attenuation processes may reduce the contaminant concentrations, but do not alter the chemical structure of the compounds. While photolysis and other chemical transformation processes can cause only minor degradation or activation of compounds, the majority of hydrocarbon degradation and removal from the environment is performed by microorganisms, mainly bacteria and fungi (Miller et al. 1988, Mulligan and Yong 2004).

Fungi biotransform a wide range of hydrocarbons using both extracellular peroxidases and laccases and the intracellular P-450 detoxification system. However, while fungal-bacterial interactions play an important role in the biodegradation of recalcitrant hydrocarbons or mixtures of contaminants, fungal hydrocarbon biodegradation is often non-specific, incomplete and can even result in the formation of more toxic metabolites (Cerniglia 1997).

In contrast to fungi, bacteria use specific catabolic pathways for hydrocarbon biodegradation. The pathways consist of a series of enzyme-catalyzed reactions that, in general, first convert a wide range of hydrocarbons to a limited number of common intermediates. These intermediates are then further degraded using a small number of metabolic pathways and channelled to the central metabolism for carbon and energy assimilation (Cerniglia 1992). The pathways used for degradation depend on the bacteria. Although the enzymes involved in hydrocarbon biodegradation have relaxed substrate specificities, they are generally specific to groups of similar compounds. For instance, the naphthalene dioxygenase enzyme catalyzes the transformation of low molecular weight (LMW) PAHs, whereas alkane monooxygenase enzyme is involved in the degradation of aliphatic hydrocarbons (Resnick et al. 1996, Throne-Holst et al. 2007). Each enzyme, and thus each step in the biodegradation pathway, is encoded by a specific gene sequence, the functional gene. Generally enzymes with similar substrate specificities are encoded by closely similar functional genes (Andreoni and Gianfreda 2007). So far, only a fraction of the hydrocarbon-degrading bacteria have been characterized in the laboratory, but it is well known that high genetic diversity exists in the functional genes that are distributed over a wide range of bacterial groups (Table 1). The expression of

functional genes is regulated by the presence and availability of the degradation substrate or reaction intermediates (Phale et al. 2007).

In addition to being used as sources of carbon and energy, hydrocarbons may also serve as co-metabolic substrates. In co-metabolism, the hydrocarbons are not selectively biodegraded or used as energy sources, but instead are transformed in a non-specific enzymatic reaction together with a structurally related substrate (Horvath 1972). Although it does not seem to benefit the individual microorganisms, co-metabolism enhances the overall biodegradation rate by transforming the compound into a more easily biodegradable form, thus allowing the community to degrade a wider range of compounds (Keck et al. 1989). The HMW PAHs, for instance, are degraded by the co-metabolic action of several organisms, even though the mass transfer rate of the compound may be too low for one microorganism to acquire enough energy from the degradation process (Kanaly and Harayama 2000).

In this thesis, the focus is on the bacterial biodegradation of aliphatic and aromatic hydrocarbons under aerobic conditions.

Table 1. Genotypes and substrate specificities of some aerobic aliphatic and aromatic hydrocarbon-degrading bacteria.

Substrate	Genotype	Gene location	Host bacterium	Reference
n-Alkanes (C_6–C_{12})	*alk*	Plasmid	*Psudomonas oleovorans* GPo1	van Beilen et al. (1994)
n-Alkanes (C_{12}–C_{18})	*alk*	Chromosome	*Acinetobacter calcoaceticus* ADP1	Ratajczak et al. (1998)
n-Alkanes (C_9–C_{18})	CYP153	Chromosome	*Acinetobacter calcoaceticus* EB104	Maier et al. (2001)
n-Alkanes (C_6–C_{36})	*alk*	Chromosome	*Rhodococcus erythropolis* NRRL B-16531	Whyte et al. (2002b)
n-Alkanes (C_{10}–C_{30})	*par*	Chromosome	*Acinetobacter* sp. M-1	Tani et al. (2001)
n-Alkanes (C_{15}–C_{36})	*lad*	Plasmid	*Geobacillus thermodenitrificans* NG80-2	Feng et al. (2007)
n-Alkanes (C_{32}–)	*alm*	Chromosome	*Acinetobacter* sp. DSM 17874	Throne-Holst et al. (2007)
LMW PAHs	*nah*	Plasmid	*Pseudomonas putida* G7	Yen and Sedar (1988)
	phd	Chromosome	*Comamonas testosteroni* GZ39	Goyal and Zylstra (1997)
	nag	Plasmid	*Ralstonia* sp. U2	Fuenmayor et al. (1998)
	nah	Chromosome	*Polaromonas naphthalenivorans* CJ2	Jeon et al. (2003)
	phn	Plasmid	*Burkholderia* sp. RP007	Laurie and Lloyd-Jones (1999)
	phn	Plasmid	*Alcaligenes faecalis* AFK2	Kiyohara et al. (1990)
HMW PAHs	*nar*	Plasmid	*Rhodococcus* sp. NCIMB12038	Larkin et al. (1999)
	phd	Chromosome	*Nocardioides* sp. KP7	Saito et al. (1999)
	nid	Chromosome	*Mycobacterium* sp. PYR-1	Kim et al. (2007)
	dpf	Plasmid	*Terrabacter* sp. DBF63	Habe et al. (2004)
T (m-/p-)X	*xyl*	Plasmid	*Pseudomonas putida* mt-2	Burlage et al. (1989)
BTE (m-/p-)X	*tod*	Chromosome	*Pseudomonas putida* F1	Zylstra and Gibson (1989)
BT	*tom*	Plasmid	*Burkholderia cepacia* G4	Shields et al. (1995)
BTEX	*tbu*	Chromosome	*Ralstonia pickettii* PK01	Olsen et al. (1994)
T	*tmo*	Chromosome	*Pseudomonas mendocina* KR1	Whited and Gibson (1991)
T (m-/p-)X	*xyl*	Chromosome	*Sphingomonas yanoikuyae* B1	Kim and Zylstra (1999)
TE(o-)X	*akb*	Plasmid	*Rhodococcus* sp. DK17	Kim et al. (2004)

1.2.1 Aerobic biodegradation

Hydrocarbons are biodegraded most efficiently in the presence of oxygen. Degradation is initiated by the addition of one or two atoms from molecular oxygen to the hydrocarbon. The enzymatic incorporation of oxygen into the reduced and chemically resistant hydrocarbon increases its oxidation state, thereby rendering the compound more susceptible for further transformation (Phale et al. 2007).

The most common aerobic degradation pathway for n-alkanes is the terminal oxidation pathway initiated by the alkane monooxygenase-catalyzed oxidation of the terminal methyl group of alkane to 1-alkanol. 1-alkanol is then serially converted to aldehyde and carboxylic acid, followed by complete mineralization through the β-oxidation pathway (Figure 2) (Watkinson and Morgan 1990). Genes involved in aerobic n-alkane degradation have been characterized from a range of Gram-negative and -positive bacteria (Table 1).

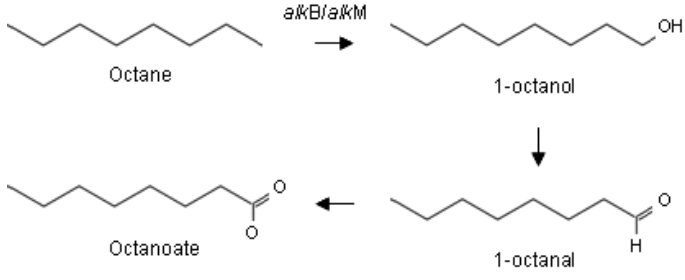

Figure 2. Monoterminal oxidation of octane to octanoate (Adapted from Watkinson and Morgan 1990).

The biodegradation of aromatic hydrocarbons can be divided into three steps: First, the aromatic ring is activated and transformed into hydroxylated aromatic metabolites including (alkyl-substituted) catechols, protocatechuic acid and gentisic acid by aromatic ring oxygenases and dehydrogenases (Step I in Figure 3). Second, the aromatic ring is opened by ring-cleavage dioxygenases to yield unsaturated aliphatic acids and aldehydes (Step II in Figure 3) which, in the third step, are used in central metabolism for energy and biomass production (Step III in Figure 3) (Peng et al. 2008, Smith 1990).

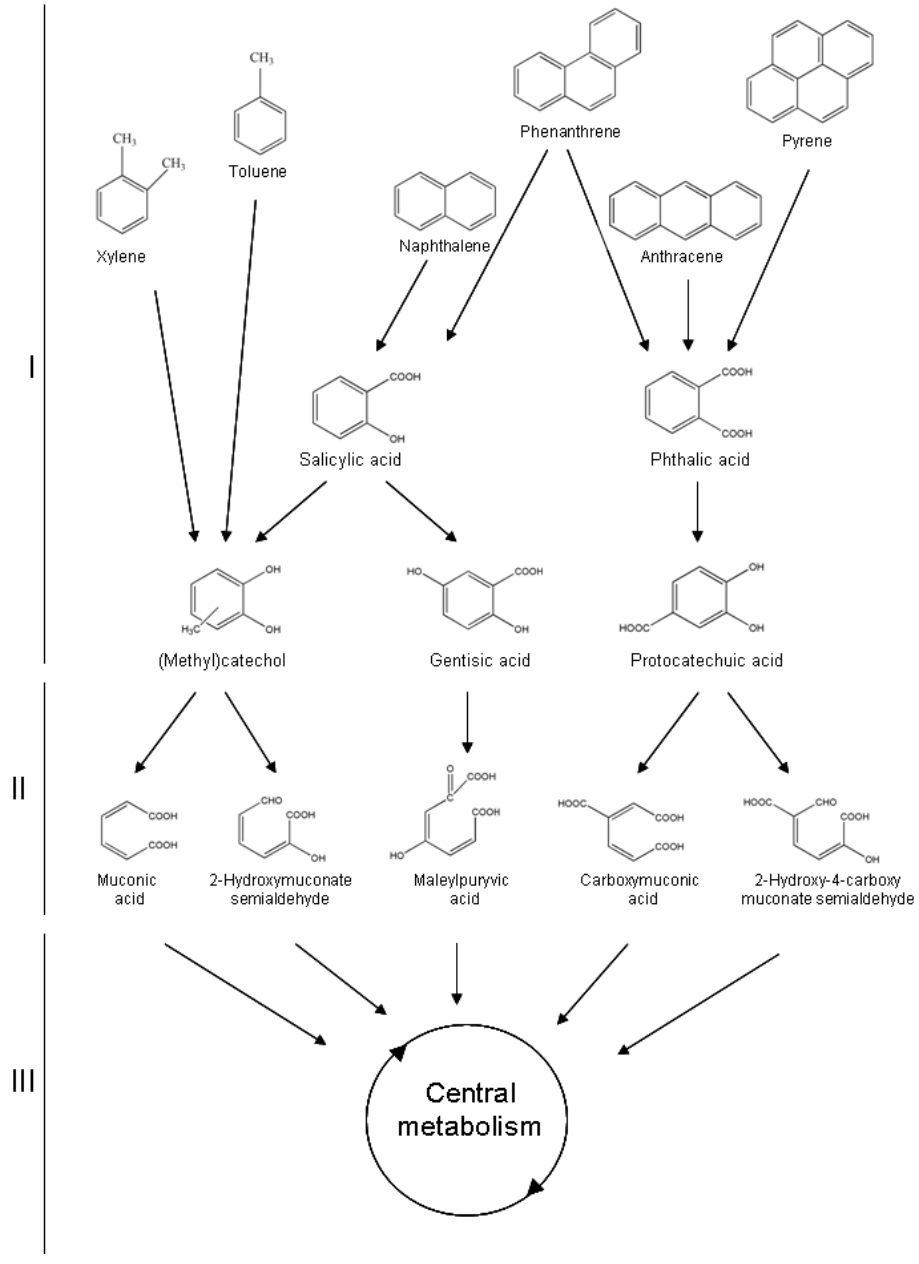

Figure 3. Aerobic biodegradation of aromatic hydrocarbons. The degradation proceeds through upper (I) and lower (II) pathways, after which the ring-cleavage products are channelled to central metabolism (III) (Adapted from Peng et al. 2008 and Smith 1990).

The initial activation of BTEX occurs either by the addition of one oxygen atom to the alkyl group of the compound by a monooxygenase enzyme or the direct hydroxylation of the aromatic ring by a mono- or dioxygenase enzyme. The subsequent reactions lead to the formation of (alkyl) substituted catechols that are converted to central metabolites via extradiol- or intradiol-ring-cleavage pathways (Smith 1990). Toluene biodegradation, for instance, can occur via several different pathways: by oxidation of the methyl group by a monooxygenase with subsequent conversion to benzyl alcohol, benzaldehyde, benzoate and catechol (Worsey and Williams 1975), by hydroxylation of the aromatic ring by toluene 2,3-dioxygenase to cis-toluene 2,3-dihydrodiol and futher conversion to methyl catechol (Zylstra and Gibson 1989), or by monooxygenation by toluene *ortho*-, *meta*- or *para*-monoxygenase enzymes to corresponding cresols that are further degraded via benzyl alcohol, methyl catechol or 4-hydroxybenzoate pathways (Olsen et al. 1994, Shields et al. 1995, Whited and Gibson 1991). Xylene isomers are degraded in a similar manner to toluene by first hydroxylation of the methyl group to methylbenzyl alcohols, followed by serial conversion to tolualdehydes, toluic acids and methyl catechols (Davey and Gibson 1974). BTEX-degrading bacteria include strains of *Pseudomonas, Burkholderia, Ralstonia, Sphingomonas and Rhodococcus* (Table 1).

The majority of LMW PAH-degrading bacterial isolates belong to the genus *Pseudomonas*, but degradation has also been demonstrated for strains of *Ralstonia*, *Sphingomonas* and *Burkholderia* (Table 1). In pseudomonads, the biodegradation of naphthalene, the smallest of the PAHs, is initiated by a naphthalene dioxygenase-catalyzed activation reaction that produces naphthalene 1,2-dihydrodiol (Resnick et al. 1996). The dihydroxylated intermediate is then rearomatized and ring-cleaved to produce salicylic acid and, further, catechol. Catechol is either *meta*-cleaved using catechol 2,3-dioxygenase or *ortho*-cleaved by catechol 1,2-dioxygenase enzymes (Figure 4) (Yen and Serdar 1988). Phenanthrene, a three-ring PAH, is first converted to 3,4-dihydrodiol, which is further degraded to 1-hydroxy-2-naphtoic acid, phthalate and protocatechuic acid or dihydroxylated to 1,2-dihydroxynaphthalene, which is degraded via the naphthalene pathway to chatecol (Barnsley 1983, Evans et al. 1965).

The metabolism of HMW PAHs containing four or more aromatic rings has been described in isolates of *Mycobacterium, Rhodococcus* and *Gordona* (Table 1). Similar to LMW PAHs, their biodegradation starts with dioxygenation (Kanaly and Harayama 2000). For instance, in *Mycobacterium* sp. PYR-1 pyrene is degraded via two dioxygenation reactions and ring-cleavage to

3,4-dihydroxyphenanthrene and, further, to *o*-phthalate in a similar way to the phenanthrene catabolic pathway (Kim et al. 2007). The PAHs which contain more than five rings, such as benzo[a]pyrene, are degraded co-metabolically (Kanaly and Harayama 2000).

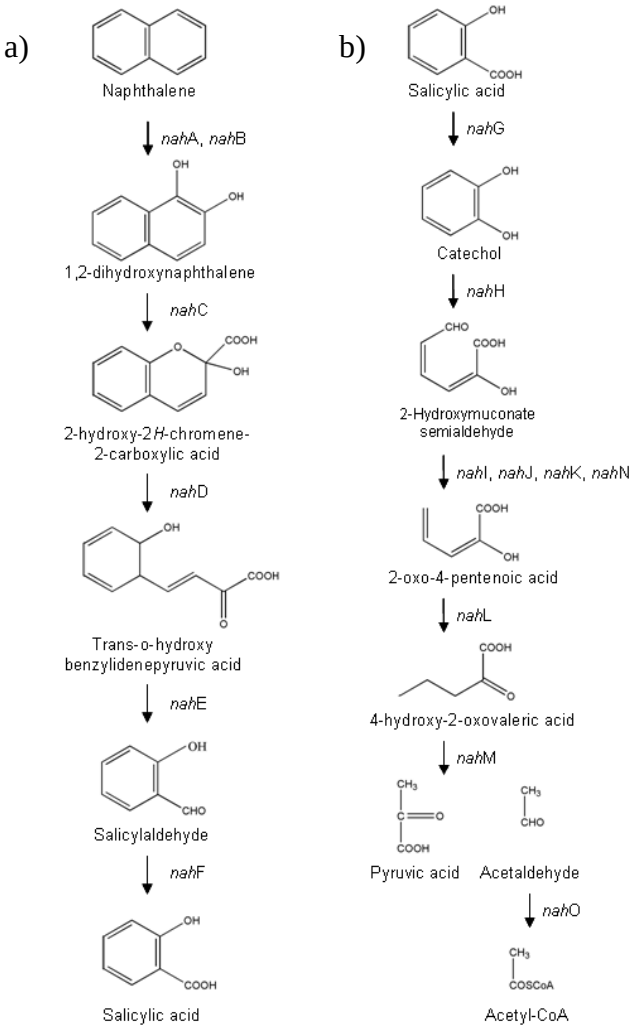

Figure 4. Genes involved in naphthalene biodegradation in *Pseudomonas putida* G7. a) Upper pathway. b) Lower Pathway. (Adapted from Peng et al. 2008.)

1.2.2 Anaerobic biodegradation

The anaerobic mechanisms of hydrocarbon biodegradation are not as well understood as the aerobic ones. Under anaerobic conditions, nitrate, manganese (IV), iron (III), sulphate or carbon dioxide serve as terminal electron acceptors (Anderson and Lovley 1997). Because the energy yield obtained from anaerobic biodegradation processes is much lower than that produced in aerobic processes, the biodegradation and growth rates under anaerobic conditions are also slower (Madigan et al. 2003).

The anaerobic n-alkane biodegradation mechanisms differ in metabolically and phylogenetically diverse microorganisms: In a denitrifying isolate, alkane activation is believed to occur through the addition of fumarate, whereas in a sulphate-reducing isolate the initial activation involves carboxylation (Rabus et al. 2001, So et al. 2003).

The anaerobic, aromatic hydrocarbon-degrading bacteria generally use the same strategy as the aerobic ones. The diverse compounds are first degraded to more common intermediates, after which the aromatic ring is cleaved and the noncyclic intermediates are converted to central metabolites. The anaerobic degradation of toluene and xylene isomers occurs via the fumarate-addition pathway that leads to the formation of benzoyl-CoA, followed by ring-cleavage and oxidation to acetyl-CoA and CO_2 (Chakraborty and Coates 2004). The fumarate addition is catalyzed by benzyl-succinate or methylbenzyl-succinate synthase (Leuthner et al. 1998). In naphthalene and phenanthrene metabolism, the initial activation occurs through carboxylation to form the central intermediates, 2-naphthoic or phenanthroic acid. The aromatic ring of the acid is then reduced to a hydroxylated intermediate, followed by ring cleavage and central metabolism (Meckenstock et al. 2004).

1.2.3 Factors affecting hydrocarbon biodegradation

Interaction between hydrocarbons and the environmental matrix is one of the most important determinants of hydrocarbon biodegradation efficiency in the environment because it affects their concentration and bioavailability. These interactions are dependent on the characteristics of the environment (particle size, organic matter content and cation exchange capacity), age of the contamination and the chemical characteristics of the hydrocarbon (water solubility, molecular topology and molecular weight) (Semple et al. 2003). For example, the

biodegradability of PAHs and n-alkanes decreases with increasing molecular size, and PAHs containing more than three aromatic rings are not readily biodegraded (Cerniglia 1992, Watkinson and Morgan 1990). Furthermore, in environments that contain high amounts of organic matter, adsorption to soil organic matter regulates the bioavailability of the hydrophobic PAHs (Weissenfels et al. 1992).

On the other hand, if the mass-transfer rate of a hydrocarbon is high, it is not the bioavailability of the hydrocarbon that limits biodegradation but the ability of the degradative microbes to metabolize it (Shuttleworth and Cerniglia 1995). Thus, the biodegradability is also defined by the population size and activity of contaminant-degrading bacteria. This, in turn, is affected by temperature, pH, moisture, soil texture, availability of electron donors, acceptors and nutrients, predation, microbial mobility and competition for substrates, as well as the nature, toxicity, concentration, distribution and surface area of the hydrocarbons (Leahy and Colwell 1990). By degrading contaminants the microbes also change the environmental conditions which, in turn, change the community composition. This can be seen in aquifer plumes, where the depletion of dissolved oxygen resulting from biodegradation leads to a succession of different redox processes (Anderson and Lovley 1997).

1.3 Ecology of hydrocarbon biodegradation

A hydrocarbon biodegradation potential is common in both contaminated and uncontaminated environments (Johnsen and Karlson 2005, Margesin et al. 2003, Saul et al. 2005, Whyte et al. 2002a). In environments containing low levels of hydrocarbons, the bacteria capable of hydrocarbon biodegradation often thrive as minor members of the microbial communities. When contamination occurs, the hydrocarbon-degrading bacteria that can assimilate the available carbon to biomass become enriched in the microbial community (Hamamura et al. 2006, Katsivela et al. 2005, Mesarch et al. 2004, Sei et al. 2003, Stapleton and Sayler 2000). Once the bioavailable carbon source is depleted, the microbial community rapidly adapts to new sources of carbon and energy and the relative proportion of the population responsible for contaminant biodegradation decreases (Hamamura et al. 2006, Powell et al. 2006, Stapleton and Sayler 2000). If the environment is contaminated by a mixture of contaminants, the microbial community changes in a new direction due to the sequential utilization of different compounds and other populations that are responsible for their

degradation become enriched (Kaplan and Kitts 2004, Powell et al. 2006, Sei et al. 2003, Viñas et al. 2005).

In environments that have previously been contaminated, the structure of the community has already changed as a result of the selection caused by contamination: In comparison to pristine environments, in contaminated environments overall loss of diversity (Saul et al. 2005), higher hydrocarbon biodegradation potentials (Johnsen and Karlson 2005) and greater numbers of contaminant-degrading bacteria (Baldwin et al. 2008, Johnsen and Karlson 2005, Sanseverino et al. 1993), as well as faster growth responses following contaminant addition (Jonhsen et al. 2007), are commonly measured. In some of these environments, the abundance of contaminant-degraders may also correlate with the abundance of bioavailable hydrocarbons (Baldwin et al. 2008, Sanseverino et al. 1993).

Multiple carbon sources are available for bacterial biodegradation at sites polluted with mixtures of contaminants. Individual microbial populations can usually metabolize only a limited range of substrates although some bacteria may carry multiple genes with different substrate specificities (Tani et al. 2001). Therefore mixed populations with different degradation capabilities are required for the degradation of mixtures of hydrocarbons (Bouchez et al. 1995, Sei et al. 2003). Even different bacteria that are capable of biodegradation of the same compounds, but carry out the degradation under different environmental conditions and substrate concentrations, may be required (Cavalca et al. 2004, Ghiorse et al. 1995). The order in which the compounds are biodegraded depends on their bioavailability: the more easily available the compound the more rapidly it is transformed (Hamamura et al. 2006).

Microbes also prefer the simple, more easily degraded hydrocarbons, such as the short chain length n-alkenes and LMW PAHs, over the more recalcitrant and complex carbon sources, including the long chain length n-alkenes and HMW PAHs (Hamamura et al. 2006, Katsivela et al. 2005, Viñas et al. 2005). Furthermore, different bacteria participate in the different phases of degradation (Hamamura et al. 2006, Kaplan and Kitts 2004, Katsivela et al. 2005, Viñas et al. 2005). In general, the more easily available and biodegradable hydrocarbons are first metabolized by fast-growing r-strategists such as bacteria in genus *Pseudomonas* or *Sphingomona*s after which the slow-growing, more stable K-strategist, such as Actinobacteria, degrade the less bioavailable compounds (Johnsen et al. 2007, Kaplan and Kitts 2004, Leys et al. 2005, Margesin et al. 2003). However, the removal of one compound may also be inhibited by another

compound in the mixture (Bouchez et al. 1995), and the preferential biodegradation of the more bioavailable fractions can leave the more recalcitrant hydrocarbons in the environment (Shuttleworth and Cerniglia 1995).

Bacteria have different genetic and physiological mechanisms for adapting to the accumulation of hydrocarbons in the environment. Hydrocarbon-degradative genes often reside in mobile genetic elements, such as conjugative catabolic plasmids and catabolic transposons, that can be transferred to other bacteria via horizontal gene transfer (Table 1) (Tan et al. 1999). Novel biodegradation mechanisms are also constantly evolving through genetic rearrangements, recombination and transposition, or by point mutations (van der Meer et al. 1992). In order to increase the mass transfer rate of hydrocarbon into the bacterial cell, some bacteria form biofilms on solid hydrocarbons or produce biosurfactants that increase their access to substrates and make the hydrophobic contaminants more bioavailable (Johnsen et al. 2005). The hydrophobic cell wall in some hydrocarbon-degrading bacteria is believed to aid in adhesion to poorly water soluble substrates (Watkinson and Morgan 1990).

1.4 Monitoring bioremediation

The bioremediation strategies available for environmental clean-up can be divided into passive and active measures. Monitored natural attenuation, which is the passive approach, is based on the long-term monitoring of *in situ* biodegradation by the intrinsic microbial communites present at the contaminated site (Rittmann 2004). In active, engineered bioremediation, on the other hand, the activity and growth of contaminant-degrading microbes, and thus also contaminant biodegradation, are stimulated by the addition of electron donors or acceptors, nutrients and/or contaminant-degrading organisms, optimization of the environmental conditions, enhancement of the bioavailability of recalcitrant hydrocarbons or a combination of these (Jørgensen et al. 2000, Menendez-Vega et al. 2007). Methods are required for evaluating the efficiency of different remediation measures on the biodegradability of specific contaminants, understanding the response of the contaminant-degrading microbial populations to the remediation measures, and optimizing the performance of the process. Monitoring biodegradation is especially important in the case of monitored natural attenuation, where the authorities require that the biodegradability of contaminants must be demonstrated (Rittmann 2004).

1.4.1 Physico-chemical monitoring methods

Chemical analysis methods play an essential role in all stages of bioremediation; site characterization, monitoring the efficiency of decontamination and evaluation of the end-point of remediation (Sarkkila et al. 2004). The standardized methods are sensitive and allow the determination of individual compounds in complex mixtures of hydrocarbons. Together with the measurement of stable isotope ratios of residual contaminants or degradation intermediates, the contaminant concentrations are also useful in evaluating the occurrence and extent of biodegradation (Griebler et al. 2004, Richnow et al. 2003).

However, these methods cannot be used to verify the complete biodegradation and detoxification of pollutants. Because the chemical analyses only describe the loss of a studied compound, the decrease in contaminant concentrations can erroneously be misinterpreted as biodegradation, even though it can be due to abiotic factors such as volatilization, adsorption and dilution or biotic transformation to other compounds that are not completely biodegraded (Shuttleworth and Cerniglia 1995). Harsh extraction techniques provide no information about contaminant bioavailability (Sabatè et al. 2006). Although the signature metabolites and isotope ratios provide more direct indication of microbial degradation activity, it may be difficult to determine where the biotransformation is actually taking place. The extent of stable isotope fractionation depends on enzymatic reactions and, similar to signature metabolites, may be difficult to interpret at sites contaminated with low concentrations of contaminants (Griebler et al. 2004, Morasch et al. 2001, Richnow et al. 2003).

1.4.2 Microbiological monitoring methods

Microcosm studies provide quantitative estimates of the ultimate aerobic biodegradability of specific compounds based on the measurement of carbon dioxide evolution (Piskonen et al. 2005, Salminen et al. 2008). Studies under controlled laboratory conditions also allow testing of the effect of different remediation measures on the biodegradation activity of intrinsic microbial communities and the degradation efficiency (Piskonen and Itävaara 2004). Selective cultivation and enumeration of contaminant-degrading bacteria with the hydrocarbon of interest as the sole source of carbon have also been used to investigate whether biodegradation potential exist at the site and to evaluate the

biodegradation efficiency of contaminants (Ferguson et al. 2007, Johnsen et al. 2007, Menendez-Vega et al. 2007).

The kinetics of contaminant biodegradation under laboratory conditions, however, differ markedly from those *in situ* because in the laboratory, the environmental samples are incubated under optimal growth conditions in the presence of high concentrations of bioavailable substrate (Rothermich et al. 2002). In addition, most environmental bacteria are not amenable to cultivation (Amann et al. 1995). This can result in the selection of fast-growing bacteria that are best adapted to laboratory conditions, but have no important role in *in situ* biodegradation and, as a result, lead to false estimates of the *in situ* biodegradation potential. Measuring soil microbial activities on the basis of soil respiration or the number of heterotrophic bacteria may also provide estimates of the efficiency of *in situ* biodegradation (Margesin et al. 2000, Menendez-Vega et al. 2007). Although soil microbial counts and activity have been observed to increase as a result of contamination (Saul et al. 2005, Viñas et al. 2005), they are indicative only of changes in the overall activity of the system. Because the increase may also occur as a result of other factors such as biodegradation of organic matter or nutrient amendment (Jørgensen et al. 2000, Margesin et al. 2003, Powell et al. 2006), the microbial activites and counts do not necessarily correlate with the numbers of contaminant-degrading bacteria or contaminant biodegradation (Salminen et al. 2008).

1.4.3 Molecular biological monitoring methods

Molecular biological methods allow the analysis of environmental microbial communities on the basis of the isolation of DNA or RNA directly from environmental samples. Because no cultivation is required, a more comprehensive picture of the composition and functionality of microbial communities in contaminated environments can be obtained.

In general, the molecular biological monitoring methods are based on polymerase chain reaction (PCR) amplification or hybridization. In PCR, the target genes are enzymatically amplified from small amounts present in environmental nucleic acid extracts to levels that can be detected. The formation or absence of amplification product is used as qualitative, end-point indicator of the presence of target gene in a given environmental sample (Traditional PCR, Figure 5 a). However, by employing quantitation standards and real-time measurement during amplification, the amount of target gene can be determined

also quantitatively (Real-time PCR, Figure 5 b). Because PCR relies on exponential amplification of target genes, it is a sensitive method for the detection of low abundance genes.

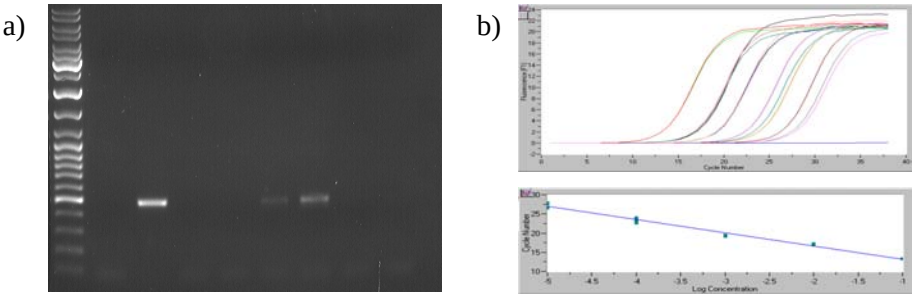

Figure 5. a) Traditional PCR using end-point analysis of PCR amplification with agarose gel electrophoresis. b) Quantitative PCR using real-time measurement of amplification and a standard curve.

In hybridization, a gene under investigation is detected with a gene probe, a single-stranded piece of DNA that is complementary to the target gene. In traditional hybridization assays, such as dot blot hydridization, several different DNA samples are bound on solid support, usually on nylon membrane, along with known amounts of target gene as a quantification standard. The target gene is then used as a probe that is labelled, generally with radioactive marker such as ^{32}P, and hybridized on the membrane. By comparing the amount of radioactivity retained in the sample DNA to the radioactivity in quantification standards, the abundance of target genes in several environmental samples can be determined at the same time (Figure 6 a).

In gene array hybridization gene probes, instead of DNA samples, are attached on a solid support, and the sample DNA under investigation is labelled and hybridized on the array. In microarray analysis the support is a glass slide and sample DNA is labelled with fluorescent dye, whereas in macroarray analysis nylon membranes and radioactive labelling are used. Gene probes to which sample DNA has hybridized produce fluorescent or radioactive signal. This indicates the presence of the respective genes in the investigated sample DNA (Figure 6 b). Since numerous gene probes can be attached on an array, gene array hybridization allows several genes to be simultaneously analyzed from one environmental sample. However, quantitative comparison between several samples is challenging.

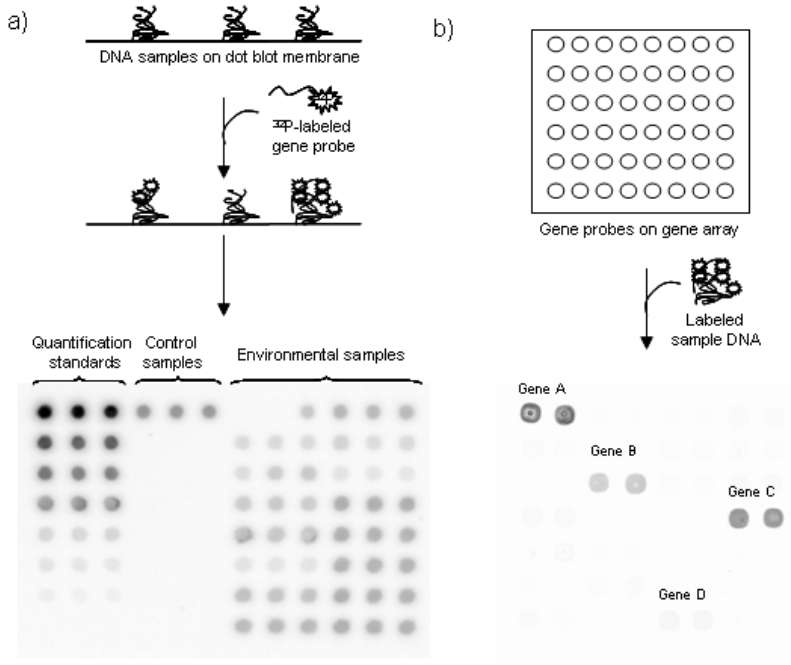

Figure 6. a) Dot blot hybridization. b) Gene array hybridization.

Depending on the type of information required, the PCR and hybridization based assays can be targeted either at phylogenetic genes such as the 16S rRNA gene encoding the small subunit ribosomal RNA that is ubiquitous in all cellular organisms, or at functional genes involved in specific metabolic processes.

1.4.3.1 Microbial community composition determination using phylogenetic genes

The analysis of phylogenetic genes with whole community fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) can be used to identify qualitative differences in microbial community composition in environmental samples collected from pristine and contaminated locations, as well as during bioremediation treatment (Ferguson et al. 2007, Viñas et al. 2005). This allows monitoring the enrichment of specific bacterial populations in response to contamination or bioremediation measures. Based on the chemical analysis of contaminant concentrations, the enriched microbial populations can then be linked to different phases of contaminant biodegradation (Kaplan and Kitts 2004, Katsivela et al. 2005), associated with a certain phylogenetic group or

potentially even linked to the biodegradation of specific compounds (Hamamura et al. 2006, Hayes and Lovley 2002). However, although it is possible to design assays for a specific group of bacteria known to be involved in the degradation of specific hydrocarbons (Leys et al. 2005, Wang et al. 1996), linking the bacterial phylogeny with a specific biodegradation function is difficult. Community fingerprinting provides information only about the dominant populations (Bent and Forney 2008). The biodegradation potential is not necessarily limited to a specific group of bacteria because of the possible occurrence of horizontal gene transfer (Cavalca et al. 2004). A bacterial population may also become enriched during biodegradation because it can use the reaction intermediates formed during biodegradation for growth (Da Silva and Alvarez 2007).

1.4.3.2 Microbial community function determination using functional genes

Functional genes provide more direct information about the functionality of microbial communities in the environment. The presence or absence of genes involved in the degradation of specific compounds in hydrocarbon-contaminated environments can be used as an indicator of the biodegradation potential of the respective compounds, and their bioavailability and transport in the environment (Marlowe et al. 2002, Mesarch et al. 2004, Stapleton and Sayler 2000, Tuomi et al. 2004). Changes in the amount of degradation genes have been shown to correlate with a decrease in contaminant concentrations as well as the efficiency at which the hydrocarbons are mineralized (Fleming et al. 1993, Park and Crowley 2006, Salminen et al. 2008). In some PAH-contaminated soils and sediments, the amount or expression of naphthalene-degradative genes has also been correlated with naphthalene concentrations (Cébron et al. 2008, Dionisi et al. 2004). Therefore the functional genes are also useful in monitoring the dynamics of contaminant-degrading bacteria in microcosms (Ringelberg et al. 2001, Sei et al. 2003) and, more recently, also in evaluating the effect of different bioremediation treatments on hydrocarbon biodegradability (Baldwin et al. 2008, Cavalca et al. 2004, Ferguson et al. 2007, Powell et al. 2006). The genes commonly used as indicators of aerobic hydrocarbon biodegradation are those involved in the initial oxidation of hydrocarbons, such as alkane monooxygenase (Powell et al. 2006), naphthalene dioxygenase (Park and Crowley 2006) and toluene monooxygenase genes (Baldwin et al. 2003), or in the ring-cleavage of common reaction intermediates such as catechol dioxygenase gene (Mesarch et al. 2004).

1.4.3.3 Gene array analysis

In order to better understand the dynamics of mixed populations involved in the degradation of complex mixtures of contaminants, several different biodegradation activities should be monitored simultaneously. Although the current PCR-based techniques can be used for the simultaneous analysis of a small number of genes (Baldwin et al. 2003), it is not feasible to analyse the large number of different genes required for the efficient degradation of complex mixtures of contaminants with these techniques.

Gene array technology enables the simultaneous analysis of thousands of genes in a single assay. After the application of 16S rRNA gene probes for studying nitrifying bacteria in pure cultures by Guschin et al. (1997), gene array platforms for microbial community profiling and for the detection of bacteria with specific biochemical functions have been developed and evaluated in a variety of environments (Table 2). Phylogenetic gene arrays containing up to 500 000 ribosomal RNA gene-targeted probes have been used to investigate microbial community composition in e.g. soil (Kyselková et al. 2008), air (Brodie et al. 2007, Wilson et al. 2002,), freshwater sediments (Peplies et al. 2006), compost (Franke-Whittle et al. 2008), wastewater (Lee et al. 2006), activated sludge (Kelly et al. 2005), acid mine drainage (Yin et al. 2007), cyanobacterial mats and acidic fens (Loy et al. 2002, Loy et al. 2004). Although the gene probes can be designed to cover the whole, currently known microbial diversity and to provide information on several levels of taxonomic specificity (Brodie et al. 2006), the design of species-specific probes and the specific detection of closely related bacteria are challenging (Franke-Whittle et al. 2008, Sanguin et al. 2006). Therefore, the phylogenetic gene arrays have also been designed to target a specific phylogenetic group such as Rhodococcales (Loy et al. 2005), Alphaproteobacteria (Sanquin et al. 2006) and actinomycetes (Kyselková et al. 2008), or a certain taxon sharing the same physiology such as sulphate reduction (Loy et al. 2002) and nitrification (Kelly et al. 2005).

The functional gene array applications involve nutrient cycling, biodegradation and risk assessment (Table 2) (Bodrossy et al. 2003, Denef et al. 2003, Iwai et al. 2007, Iwai et al. 2008, Lee et al. 2006, Moisander et al. 2006, Rhee et al. 2004, Steward et al. 2004, Stralis-Pavese et al. 2004, Taroncher-Oldenburg et al. 2003, Tiquia et al. 2004, Yin et al. 2007. During the last ten years, the studies have focused on the development of small-scale functional gene arrays for method validation or microbial community profiling in laboratory enrichments

and environmental samples (Table 2). The number of probes attached on environmental gene arrays has increased gradually. The most comprehensive functional gene array developed to date contains more than 24 000 functional gene probes targeting more than 10 000 genes involved in organic compound biodegradation, metal resistance and sulphur, nitrogen and carbon cycles (He et al. 2007). It has been used to address questions ranging from *in situ* bioremediation of uranium-contaminated groundwater to nutrient cycling in Antarctic soils (He et al. 2007, Yergeau et al. 2007).

The gene array technology holds great promise also for bioremediation. Both phylogenetic and functional gene arrays have demonstrated that differences in microbial community composition and functionality are linked to the extent or type of contamination (He et al. 2007, Neufeld et al. 2006, Rhee et al. 2004, Yin et al. 2007). The functional genes involved in the biodegradation of benzene, polychlorinated biphenyl, naphthalene and other organic contaminants as well as in metal reduction, have been successfully detected in samples recovered from microcosm studies and oil-, PAH-, BTEX- and heavy metal-contaminated soil and groundwater samples (Denef et al. 2003, Iwai et al. 2008, Rhee et al. 2004, Wu et al. 2006). Marked changes have also been detected in the microbial community composition and functionality following contaminant biodegradation or biostimulation in laboratory microcosms (Chandler et al. 2006, Rhee et al. 2004).

However, thus far the environmental gene array applications have focused primarily on profiling microbial communities and functions in different environmental samples or on the characterization of the differences in community composition in a limited number of environments samples. Applications in which bacterial dynamics are monitored during different phases of microbial processes are limited. In laboratory-scale enrichments, for instance, only the end-point of contaminant degradation has been analyzed without demonstrating the actual biodegradation of contaminants (Denef et al. 2003, Iwai et al. 2008, Rhee et al. 2004). In addition to the small-scale gene expression study on resin acid biodegradation in pulp mill effluent-treating bioreactor that was reported by Dennis et al. (2003), monitoring of bacterial dynamics during different process phases has been restricted to uranium biotransformation in soil columns and *in situ* bioremediation of uranium-contaminated groundwater and sediment (Brodie et al. 2006, Chandler et al. 2006, He et al. 2007). Little information is available on the feasibility of functional gene arrays as tools for monitoring hydrocarbon biodegradation in field-scale bioremediation processes.

Table 2. Some phylogenetic and functional gene array applications in environmental microbiology and biotechnology.

Target organism / function	Probe length (bp)	Number of probes	Sensitivity	Specificity	Application	Reference
Phylogenetic gene arrays						
Prokaryotes and Eukaryotes	20	62 358			Air	Wilson et al. 2002
Sulphate-reducing bacteria	18	132	50 ng PCR product		Tooth pocket and hyper saline cyanobacterial mat	Loy et al. 2002
					Low-sulfate, acidic fen soil	Loy et al. 2004
Rhodocyclales	18	79	1 %[a] (PCR)[b]		Activated sludge from industrial wastewater treatment plant	Loy et al .2005
Pathogens and compost-originating microbes	17–25	65	5 %		Compost	Franke-Whittle et al. 2005
		369	(PCR)			Frnake-Whittle et al. 2008
Ammonia- and nitrite oxidizing bacteria	15–22	8	2 %		Activated sludge	Kelly et al. 2005
Metal- and sulphate-reducing bacteria	17–25	71			Uranium-contaminated sediment	Chandler et al. 2006
Freshwater bacteria	20	70	2 %		Freshwater sediment	Peplies et al. 2006
Most abundant phylotypes in hexachlorocyclohexane-contaminated soils	35	112	0.5–5 % (PCR)		Hexachlorocyclohexane-contaminated soils	Neufeld et al. 2006
Bacterial pathogens	20–28	24	10^2 gene copies (no background)[c]		Raw sewage and final effluent from municipal wastewater treatment plant	Lee et al. 2006
Alphaproteobacteria	20	122	8.4 %		Maize rhizosphere	Sanguin et al. 2006
Bacteria and Archaea	25	506 944	10^6 (PCR)		Uranium-contaminated soil column	Brodie et al. 2006
					Air	Brodie et al. 2007

Target organism / function	Probe length (bp)	Number of probes	Sensitivity	Specificity	Application	Reference
Acidophiles	50	1071 (571)	5 ng genomic DNA (no background)		Acidic environments and bioleaching system	Yin et al. 2007
Actinomycetes	18–21	113			Forest soil	Kyselková et al. 2008
Functional gene arrays						
Nutrient cycling						
Nitrogen cycling	330–2 300	104	25 ng community DNA	80–85 %	Marine sediment and soil	Wu et al. 2001
Nitrogen cycling	70	64	10^7 gene copies (PCR)	87 %	River sediment	Taroncher-Oldenburg et al. 2003
Methanotrophy	16–26	59	5 % (PCR)		Landfill site and CH_4-amended soil microcosm	Bodrossy et al. 2003
Methanotrophy and ammonia oxidation	18–27	68			Lysimeters simulating landfill site	Stralis-Pavese et al. 2004
					Lysimeters simulating landfill site	Bodrossy et al. 2006
Nitrogen cycling, methane oxidation and sulphate reduction	50	763	60 ng genomic DNA	< 86 %	Sediment	Tiquia et al. 2004
Nitrogen fixation	318–333	83	13 pg (PCR)	78–88%	River water	Steward et al. 2004
Nitrogen fixation	60	96		>77 %	Nutrient-amended microbial mat	Moisander et al. 2006
Carbon, nitrogen, sulphur and iron metabolism, metal resistance and other functions associated with acidophiles	50	1 071 (501)	5 ng genomic DNA (no background)		Acid main drainage and bioleaching system	Yin et al. 2007

Target organism / function	Probe length (bp)	Number of probes	Sensitivity	Specificity	Application	Reference
Biodegradation						
2,4-dicholorophenoxyacetic acid and resin acid degradation	271–1 300	26	10^5 cells ml^{-1}	70 %	Resin acid-induced pulp mill bioreactor culture	Dennis et al. 2003
Polychlorinated biphenyl- and chlorobenzoate degradation	70	10	1 % (TSA)[d]		Polychlorinated biphenyl-amended sediment microcosm	Denef et al. 2003
Biodegradation of organic contaminants and metal resistance	50	1662	5 %	88 %	Naphthalene-enriched microcosm and PAH- and BTEX-contaminated soil	Rhee et al. 2004
Biodegradation of organic contaminants, metal resistance and nutrient cycling	50	2 006	1 ng community DNA (WGA)[e]		Uranium- and heavy metal-contaminated groundwater	Wu et al. 2006
Biodegradation of organic contaminants, metal resistance and nitrogen, carbon, sulphur, phosphorous cycling	50	24 243			Uranium-contaminated groundwater	He et al. 2007
Benzene degradation	60	87			Benzene-amended soil	Iwai et al. 2007
	60	148			Benzene-amended microcosms and oil-contaminated soil	Iwai et al. 2008
Risk assessment						
Virulence and pathogenity	60–70	10	10^8 gene copies (no background)		Raw sewage and final effluent from municipal wastewater treatment plant	Lee et al. 2006

[a] Percentage of the whole microbial community in the analyzed sample.
[b] The target genes were PCR amplified before hybridization.
[c] The analysis was performed with DNA isolated from pure bacterial culture.
[d] Tyramide signal amplification was used to increase analysis sensitivity.
[e] Whole genome amplification was used to increase the amount of DNA prior to hybridization.

2. Aims of the study

One of the main challenges in the development and management of more efficient biological treatment processes is to demonstrate and monitor the biodegradability of specific compounds in the environment. The aim of this thesis was to investigate the applicability of molecular biological methods as tools for biodegradation process monitoring and optimization. Aliphatic and aromatic hydrocarbons were selected as model compounds because of their ubiquitous distribution in contaminated environments.

The specific aims were:

- to investigate the applicability of the functional genes involved in aerobic bacterial metabolism of hydrocarbons as indicators of hydrocarbon biodegradation in the environment
- to develop a functional gene array targeting aliphatic and aromatic hydrocarbon biodegradative pathways for simultaneous monitoring of the biodegradation of different hydrocarbons
- to evaluate the feasibility of the developed methods in monitoring field-scale bioremediation processes.

3. Materials and methods

The materials and methods used in the current study are shortly described in this chapter. More detailed information is presented in the original publications (Papers I–IV).

3.1 Bacterial strains and DNA control samples

The bacterial strains used in this thesis are listed in Table 3. The origin of each strain and the corresponding growth conditions are described in papers I, II, III and IV.

Table 3. Bacterial strains used in this thesis.

Strain	**Codes**	**Paper**
Acinetobacter calcoaceticus ADP1	VTT E031437	IV
Bacillus subtilis	DSM 10	I II III
Burkholderia sp. RP007	ICMP 13529	I IV
Desulfosarcina variabilis	DSM 2060	IV
Escherichia coli	DSM 1576	I II III
Pseudomonas putida	DSM 291	I II III
Pseudomonas putida F1	DSM 6899	II III IV
Pseudomonas putida G7	DSM 4476	I II III IV
Pseudomonas putida mt-2	DSM 3931	II III IV
Pseudomonas putida OUS82 with minitn5(kan)-Pa1/04/03-*rfp*	VTT E042480	I
Pseudomonas sp. TF4-1L	ATCC 29347	IV
Ralstonia sp. U2	NCIMB 13827	I IV

The pSSO1genBlue, pSSO2genBlue and pCMV5-Mint1 plasmids, *Oncorhynchus mykiss* cDNA and the DNA coding for the anti-morphine Fab fragment that were used for PCR amplification of the control probes in the gene array analyses are described in papers II, III and IV.

3.2 Microcosm studies

A mixed liquid culture microcosm was established to evaluate the feasibility of the simultaneous detection of several functional genes in monitoring the biodegradation of a mixture of compounds (Paper II). *P. putida* strains F1, G7 and mt-2 were grown in mixed liquid culture, with toluene and naphthalene supplied as the sole sources of carbon. The biodegradation of toluene and naphthalene was monitored on the basis of $^{14}CO_2$ evolution. Changes in the amount of toluene and naphthalene degradation genes in DNA samples recovered during the course of the study were investigated with functional gene macroarray analysis and dot blot hybridization (Paper II).

A soil slurry microcosm was set up to investigate the effect of bacterial inoculum on PAH biodegradation efficiency in soil slurry bioreactors by Piskonen et al. (2005). The soil slurry containing 10 % of PAH-contaminated soil was amended with ^{14}C-labelled naphthalene, and biodegradation was monitored by measuring naphthalene mineralization on the basis of $^{14}CO_2$ production. The feasibility of enhancing biodegradation by means of inoculation was studied in a soil slurry spiked with naphthalene-degrading *P. putida* G7 and non-inoculated soil slurry. The biodegradation of naphthalene during the course of the study was monitored by quantitating the amount of naphthalene dioxygenase gene with real-time PCR (Paper I). In the following study, samples from the same experiment were used in the functional gene microarray analysis (Paper III).

3.3 Environmental samples

The environmental samples employed in this thesis were obtained from uncontaminated and contaminated environments, as well as from bioremediation processes treating hydrocarbon-contaminated soil and groundwater (Table 4).

Table 4. Environmental samples used in the thesis.

Sample	Contaminants	Treatment	Source	Papers
Soil	PAHs	Storage in a pile	Ekokem	I III
Soil	Tar	Composting	Soilrem	I
Soil	Diesel oil, light fuel oil	Composting, vapour extraction	Soilrem	I
Groundwater	Creosote	Air sparging, nutrient infiltration	Ramboll	IV
Soil	Diesel oil, lubrication oil	Storage in a pile, composting in a bioreactor	Merinonita	
Soil		Agricultural soil, no bioremediation	Centre Experimental Horticole de Marsillarques, France	III

3.3.1 *In situ* bioremediation of creosote-contaminated groundwater

Creosote-contaminated groundwater samples obtained from a former wood impregnation plant were used to investigate the applicability of functional gene array analysis in bioremediation process design and monitoring (Paper IV). The site was under a long-term monitoring program for the risk assessment and design of remediation strategy. A pilot-scale air sparging and nutrient infiltration treatment had previously been conducted at the site in order to evaluate the effect of aeration and nutrient amendment in enhancing intrinsic hydrocarbon biodegradation. Four months after the biostimulation, groundwater samples were recovered from six monitoring wells located along the contaminant plume, outside the plume area and directly downstream from the biostimulation treatment zone. The long-term influence of biostimulation on the intrinsic microbial communities was analyzed with a functional gene array, real-time PCR and whole community fingerprinting using DGGE, and the results were compared to microbiological and chemical monitoring data.

3.3.2 *Ex situ* bioremediation of diesel oil- and lubrication oil-contaminated soil

The feasibility of using functional gene array analysis in field-scale monitoring of bioremediation processes was also investigated during the composting of diesel oil- and lubrication oil-contaminated soil (Unpublished data). The contaminated soil, polluted as a consequence of long-term leakage from an underground storage tank, was excavated from an old bus depot. At the soil treatment facility (Merinonita Ltd, Porvoo, Finland), a subsample of 1 600 kg was stored in a pile in a shed for ten months similar to the rest of the contaminated soil. After two weeks of storage, 20 kg of fertilizer (Puutarhan Y1, Kemira-Growhow, Finland, 9 % total nitrogen and 6 % total phosphorous) and 1 m^3 of wood chips were added to the soil to enhance microbial activity. Composite samples for the analysis of microbial communities were recovered in the beginning of the study and after 2, 4, 7, 11, 15, 22 and 46 weeks of storage. At each sampling time, 8 samples were collected with a 190 cm auger and combined to form a composite sample on which all microbial and molecular analyses were performed. Samples for DNA isolation were stored at –80 °C. The soil was watered after 22 weeks because of drying (dry weight 94.5 % w/w).

After 46 weeks of storage, the soil was mixed with five kilograms of fertilizer (Puutarhan Y1, Kemira-Growhow, Finland, 9 % total nitrogen and 6 % total phosphorous) and composted for four weeks in a BRCSoil Bioreactor (223.5 cm x 204.5 cm x 550 cm; soil volume 26 m^3) (Figure 7). During treatment the soil was heated to 37 °C and aerated for one hour every three hours (pump capacity 2600 m^3 h^{-1}). During composting, CO_2 production was monitored continuously using a GMM221 Carbon dioxide Module incorporated with a Vaisala CARBOCAP Sensor (Vaisala, Finland). Composite soil samples were collected from composting process after 4, 7, 11, 14, 21 and 28 days during the four-week treatment period. Aerobic microbial activity was determined by fluorescein diacetate (FDA) hydrolysis on the same day, and soil samples for DNA isolation were stored at –80°C.

Figure 7. The BRCSoil Bioreactor used for composting the diesel oil- and lubrication oil-contaminated soil.

The hydrocarbon concentration of the soil was determined by gas chromatography in an accredited analysis laboratory following soil excavation, after 46 weeks of storage and at the end of the four-week composting period, i.e. 50 weeks after soil excavation. The chemical analysis data were obtained from Merinonita Ltd. The hydrocarbon-degrading bacteria were investigated by functional gene array, real-time PCR and dot blot hydridization analysis of alkane monooxygenase genes. Changes in the bacterial community composition were monitored by PCR-DGGE analysis of 16S rRNA gene fragments.

3.4 Monitoring methods

A combination of physico-chemical, microbiological and molecular biological methods were used in this thesis to investigate contaminant concentrations and environmental parameters, microbial abundance and activity, and the structure and functionality of microbial communities in the microcosm studies and hydrocarbon-contaminated environmental samples (Table 5).

Table 5. Physico-chemical, microbiological and molecular biological methods used in this thesis.

Assay	Method	Paper	Reference
Physico-chemical methods			
pH	Electrode measurement from soil slurry	I	Foster (1995)
	Potentiometric measurement	IV	SFS (1979)
Dry weight (%)	Drying at 105ºC	I	
Temperature (°C)		IV	
Electrical conductivity (mS m^{-1})	Conductiometric measurement	IV	SFS (1994)
Dissolved oxygen (mg l^{-1})	Polarographic sensor	IV	
Total organic carbon (mg l^{-1})	Infrared spectrometry	IV	SFS (1997)
Redox potential (mV)		IV	
Total nitrogen (mg l^{-1})	Flow injection analysis spectrophotometry	IV	
Ammonium nitrogen (mg l^{-1})	Photometric measurement	IV	
Total phosphorous (mg l^{-1})	Flow injection analysis spectrophotometry	IV	
Dissolved iron (mg l^{-1})	Atomic absorption spectroscopy	IV	SFS (1980)
CO_2 production (%)	GMM221 Carbon dioxide Module and Vaisala CARBOCAP Sensor (Vaisala)		
Total hydrocarbons (µg l^{-1})	GC/MS with pentane extraction	IV	
Aliphatic hydrocarbons (C_4 to C_{10})	GC/MS with methanol extraction		
Aliphatic hydrocarbons (C_{10} to C_{40})	GC/FID with acetone-heptane extraction		Piskonen et al. (2005)
PAHs (EPA16) (mg kg^{-1})	GC/MS with toluene-DMSO-hexane extraction	I	Karstensen (1996)
(mg kg^{-1})	GC/MS with acetonhexane extraction	I	
(mg l^{-1})	GC/MS with hexane extraction	IV	
Naphthalene (mg l^{-1})	GC/FID with the static headspace technique	II	
Toluene (mg l^{-1})	GC/FID with the static headspace technique	II	

Assay	Method	Paper	Reference
Microbiological methods			
Cell density	DAPI staining	IV	Kepner and Pratt 1994
Microbial activity	FDA hydrolysis	IV	Alef 1995
Mineralization of ^{14}C-labelled naphthalene and toluene	$^{14}CO_2$ production, scintillation counting	I II III	
Molecular biological methods			
DNA isolation			
Plasmid DNA	Alkaline lysis	I II III	Sambrook and Russel (2001)
Total DNA	Bead beating, isopropanol precipitation, phenol:chloroform:isoamylalcohol extraction, GeneClean Turbo DNA purification kit (Qbiogene)	I II III IV	Stephen et al. (1999)
PCR amplification			
Primer design	OligoCalc, FastPCR	I III IV	Kalendar (2007), Kibbe (2007)
Bacterial community analysis			
16S rRNA gene amplification	PCR	II IV	Muyzer et al. (1993), Nübel et al. (1996)
DGGE		I II IV	Piskonen et al. (2005), Laitila et al. (2007)
Cluster analysis of DGGE profiles	Bionumerics (Applied Maths)	IV	
Quantification of functional genes			
	Dot blot hybridization	I II III	
	Quantitative real-time PCR	I IV	

Assay	Method	Paper	Reference
Functional gene macroarray hybridization			
Preparation of gene probes	PCR	II	
Matrix	Nylon membrane	II	
DNA labelling	Random labelling with ^{32}P	II	
Signal detection	Storage phosphorescence	II	
Functional gene microarray hybridization			
Preparation of gene probes	PCR	III IV	
Matrix	Poly-L-lysine- or aminosilane-coated glass slide	III IV	
DNA labelling	Random labelling with Cy3	III IV	
Signal detection	DNA microarray scanner	III IV	
Sequence analysis			
Cloning	TOPO TA Cloning Kit (Invitrogen)	I	
Sequencing	BigDye® Terminator v3.1 Cycle Sequencing kit and capillary electrophoresis (Applied Biosystems)	I III	
Sequence analysis	Chromas Version 2.13 (Technelysium)	I III	
Sequence alignment	Clustal W, ARB	I III	Higgins et al. (1994) Ludwig et al. (2004)
Phylogenetic analysis	ARB, BLAST, Fasta3	I III IV	Altschul et al. (1997) Pearson and Lipman (1988) Ludwig et al. (2004)

4. Results and discussion

4.1 Monitoring naphthalene biodegradation by a functional gene

The aerobic biodegradation of organic compounds is generally determined on the basis of the CO_2 produced as a result of mineralization (Battersby 1997). In order to evaluate the validity of functional genes as indicators of hydrocarbon biodegradation, biodegradation of naphthalene was first studied in microcosms by using ^{14}C-labelled naphthalene as an indicator of naphthalene biodegradation and naphthalene dioxygenase (*nah*A) gene as a marker gene (Piskonen et al. 2005).

It was possible, using the naphthalene dioxygenase gene as a functional marker, to monitor naphthalene biodegradation (Paper I). The amount of the naphthalene dioxygenase gene copies increased with increasing $^{14}CO_2$ production and remained elevated during active biodegradation. After the biodegradation ceased, the *nah*A gene abundance and, thus, proportional amount of naphthalene-degrading bacteria decreased. A similar succession was observed by Wang et al. (1996) who monitored the abundance of the 16S rRNA gene of *Mycobacterium* sp. PYR-1 during pyrene mineralization in a soil slurry microcosm.

The real-time PCR quantification of the *nah*A gene also enabled to link the differences in mineralization efficiencies between treatments to the differential growth of naphthalene-degrading bacteria (Figure 5 in paper I). The naphthalene biodegradation rate was accelerated by inoculation of naphthalene-degrading *P. putida* G7, which was demonstrated by the rapid increase in the number of naphthalene dioxygenase genes and $^{14}CO_2$ evolution. In the non-inoculated soil slurry, in contrast, an adaptation period during which a slow enrichment of intrinsic naphthalene-degrading bacteria was observed, preceeded naphthalene mineralization.

The applicability of the real-time PCR quantification of functional genes in studying hydrocarbon biodegradability was also demonstrated in tar- PAH-, oil- and diesel oil- and lubrication oil-contaminated soil samples, as well as in creosote-contaminated groundwater (Papers I and IV). In soil samples, the highest amount of naphthalene dioxygenase gene was detected in soil containing the highest concentration of aromatics (Figure 8). Although this suggested, similar to the PAH-contaminated soils and freshwater sediments investigated by Cébron et al. (2008) and Dionisi et al. (2004), that the abundance of functional genes may also be useful for evaluating the extent of contamination, the relationship between the number of functional genes and hydrocarbon concentration is not straightforward. As demonstrated in creosote-contaminated groundwater in paper IV, toxic levels of contaminants or other environmental factors may limit bacterial growth (Figure 8). According to Tuomi et al. (2004), the abundance of functional genes correlated with the naphthalene mineralization efficiency only in oxic soil layers. The amount of functional genes is also dependent of the stage of biodegradation, as demonstrated by the dynamic changes in *nah*A copy numbers during naphthalene biodegradation in soil slurry microcosm in paper I.

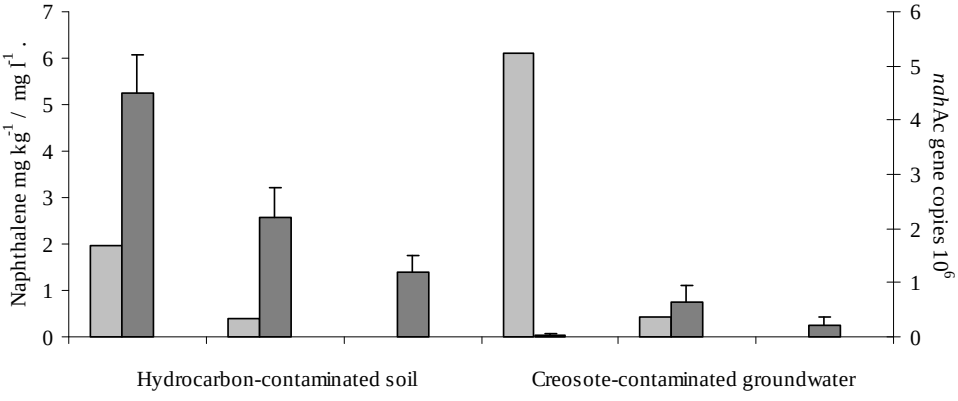

Figure 8. The naphthalene concentration (light grey) and the number of naphthalene dioxygenase gene copies (dark grey) in hydrocarbon-contaminated soil samples recovered from full-scale treatment processes (Paper I) and in the creosote-contaminated groundwater (Paper IV). The error bars represent standard deviations between three replicate measurements.

4.2 Simultaneous monitoring of naphthalene and toluene biodegradation by several functional genes

In order to simultaneously monitor the biodegradation of different hydrocarbons, a preliminary functional gene macroarray containing seven functional gene probes and four control probes was developed (Table 1 in paper II). The functional gene probes were designed to target genes involved both in the initial oxidation of aromatic ring and in ring cleavage in the aerobic BTEX and LMW PAH biodegradation (for example naphthalene-degradative *nah*A, *nah*E and *nah*H genes in Figure 4).

The hybridization signals obtained by the preliminary functional gene macroarray were in good agreement with the mineralization of specific hydrocarbons. Similar to the soil slurry microcosm studied in paper I, the hybridization signal intensity increased as ^{14}C-labelled toluene and naphthalene were actively mineralized, and decreased when $^{14}CO_2$ production ceased (Figures 1 and 3 in paper II). The consistent detection of genes from different steps of the biodegradation pathways showed that upper and lower pathways required for complete hydrocarbon biodegradation could be simultaneously detected (Figure 2 in paper II).

It was also possible to simultaneously measure the mineralization of different hydrocarbons. The high hybridization signal intensity in gene probes targeting the toluene biodegradation pathway in the beginning of the study showed that the more water soluble monoaromatic toluene was degraded faster than naphthalene, which consists of two fused aromatic rings (Figure 9 a). Once the toluene had been mineralized, increased hybridization signal intensities were observed in the naphthalene-degradative gene probes, thus demonstrating the start of naphthalene biodegradation (Figure 9 b). A similar successive degradation of different aromatic hydrocarbons was reported by Bouchez et al. (1997), who demonstrated in bacterial cultures that biodegradation of the three-ring phenanthrene was activated only after two-ring naphthalene was depleted from the medium. In their study, however, the decrease in hydrocarbon concentrations was used as an indicator of biodegradation, and no functional genes were studied.

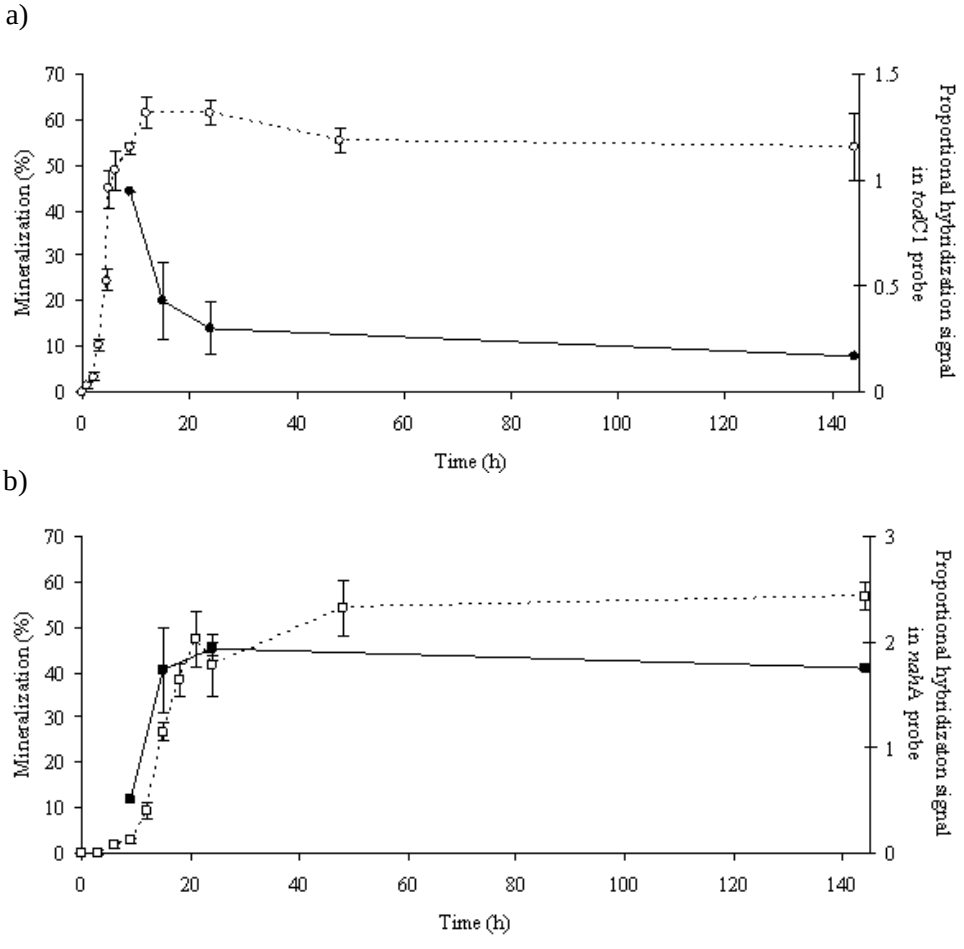

Figure 9. Toluene (a) and naphthalene (b) mineralization (dashed line) and proportional hybridization signal intensities in the toluene and naphthalene dioxygenase gene-targeting probes attached to the functional gene macroarray (solid line) in the mixed bacterial culture microcosms. The error bars represent standard deviations between three replicate microcosms.

4.3 Functional gene array analysis of naphthalene biodegradation in a soil slurry microcosm

The naphthalene- and toluene-degradative gene probes were then attached on a glass slide-based, small-scale functional gene microarray. According to Bodrossy et al. (2003) and Wu et al. (2001), gene probes with identical melting

temperatures but different lengths may result in different hybridization efficiencies. Although no significant differences were observed between the signal intensities in the *xyl*A (2214 bp) and *xyl*E (250 bp) gene probes on the functional gene macroarray (Figure 2 in paper II), the naphthalene- and toluene-degradative gene probes were optimized to have as similar length as possible (Table 1 in paper III). A hybridization signal normalization method based on an internal standard probe was also optimized in order to enable direct comparison of hybridization signal intensities between different samples and process time points (Paper III).

The analysis was validated with samples containing naphthalene-degrading *P. putida* G7 DNA mixed with agricultural soil DNA and samples obtained from the non-inoculated, PAH-contaminated soil slurry microcosm (Piskonen et al. 2005). In all the samples the optimized array analysis reproducibly detected target genes in the presence of complex background DNA. In the soil slurry microcosm, the hybridization signal intensities in the naphthalene-degradative gene probes were also in good agreement with naphthalene mineralization. Higher normalized signal intensities were detected in the naphthalene-degradative gene probes during the active phase of naphthalene mineralization than in the end of the study, when naphthalene degradation had ceased (Figure 5 in paper III). The consistent detection of the three genes involved in different steps in the naphthalene biodegradation pathway demonstrated that naphthalene was most likely mineralized via the catechol intermediate.

The results show that functional gene microarray analysis is applicable for monitoring the growth of hydrocarbon-degrading bacteria during hydrocarbon biodegradation in laboratory-scale microcosm experiments. In contrast to a previous study, in which a functional gene array was used to detect changes in microbial community functionality before and after naphthalene biodegradation, our results demonstrate that monitoring the bacterial dynamics during hydrocarbon mineralization provides information on the actual biodegradability of hydrocarbons (Rhee et al. 2004). However, this is only restricted to cases in which the hydrocarbons are used as growth substrates to increase the number of the respective degraders.

4.4 Field-scale monitoring of hydrocarbon biodegradation

The applicability of functional gene array analysis in field-scale bioremediation was evaluated in two bioremediation processes: *in situ* bioremediation of

creosote-contaminated groundwater (Paper IV), and *ex situ* bioremediation of diesel oil- and lubrication oil-contaminated soil (Unpublished data). In order to investigate the effect of sequence similarity on hybridization specificity and to expand the target range of the array, five gene probes targeting LWM PAH biodegradation and three gene probes targeting n-alkane biodegradation were added to the functional gene microarray.

4.4.1 *In situ* bioremediation of creosote-contaminated groundwater

At the creosote-contaminated site investigated in paper IV, a pilot-scale remediation using air sparging and nutrient infiltration had previously been performed in order to evaluate aeration and nutrient amendment as a remediation method. At this site, the functional gene array analysis revealed intrinsic aerobic PAH biodegradation potential along the creosote-plume (Figure 8). However, because no marked decrease in PAH concentrations had been observed during the two-year monitoring period, the results suggested that the aerobic PAH biodegradation was limited by the low concentrations of dissolved oxygen in the untreated groundwater. The aerobic biodegradation of PAHs could, nevertheless, be enhanced by air sparging and nutrient infiltration, as shown by the four times higher hybridization signal intensity in the naphthalene dioxygenase-targeting gene probe in the biostimulated area than in the monitoring well located upstream from it (Figure 10).

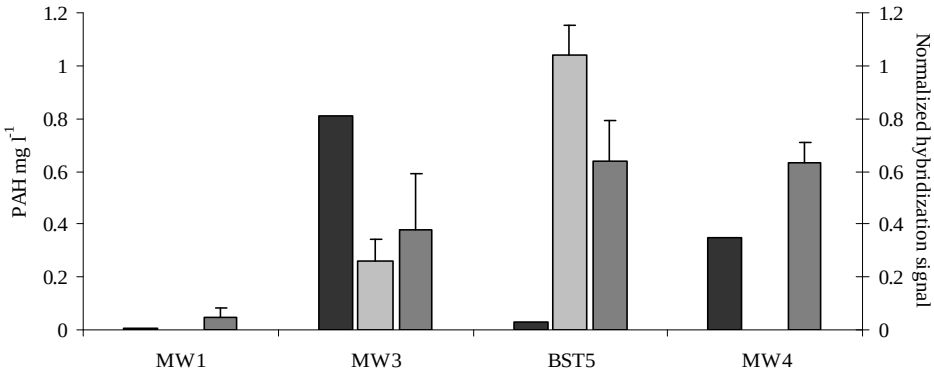

Figure 10. Total PAH concentration (dark grey) and normalized hybridization signal intensities in the naphthalene dioxygenase (light grey) and gentisate dioxygenase (medium grey) gene probes in groundwater samples obtained from a creosote-contaminated site. The samples were recovered from uncontaminated reference well (MW1), monitoring well located downstream from the hot spot and upstream from the zone treated by airsparging and nutrient infiltration (MW3), monitoring well located in the bioactivated area, downstream from the injection wells (BST5), and monitoring well located downstream from the bioactivated area (MW4). The error bars represent standard deviations between three replicate hybridizations.

Combining molecular biological monitoring methods with chemical and microbiological data thus proved valuable in understanding the factors affecting PAH biodegradation in the groundwater samples. In the monitoring wells in which anoxic conditions limited biodegradation, PCR-DGGE analysis and FDA hydrolysis showed reduced bacterial community diversity, enrichment of sulphate-reducing and denitrifying bacteria, as well as low aerobic microbial activity. In the biostimulated area, on the other hand, the molecular biological and microbiological data demonstrated that the decrease in total PAH concentrations observed during treatment by chemical analysis methods was due to increased aerobic microbial biodegradation, and not to volatilization or other abiotic processes.

Moreover, simultaneously with the naphthalene dioxygenase gene, the functional gene microarray detected increased amount of PAH-biodegradative gentisate dioxygenase gene in both the biostimulated area and the monitoring well located downstream from it (Figure 10). This showed that, in addition to the microcosms investigated in paper II, the functional gene array analysis is also applicable for the simultaneous monitoring of several biodegradation functions in complex environmental samples. However, because the two genes most likely originate from different organisms, it is clear that further studies are needed in order to better understand the functional diversity present at the site.

4.4.2 *Ex situ* bioremediation of diesel oil- and lubrication oil-contaminated soil

Monitoring the composting process of diesel oil- and lubrication oil-contaminated soil showed that combining the molecular biological analyses with chemical, physical and microbiological monitoring methods is a valuable approach also in process monitoring and optimization (Unpublished data). According to the functional gene microarray analysis, alkane-degrading bacteria were enriched after excavation of the contaminated soil and the addition of nutrients. The normalized hybridization signal in the Mer0307 probe that targets alkane monooxygenase gene increased from 0.09 immediately after excavation to 0.42 (±0.16) after seven weeks of storage (Figure 11). This was in agreement with the dot blot and real-time PCR quantification of the alkane monooxygenase gene that showed an over ten times higher number of target genes after seven weeks of storage than after excavation (Figures 11 and 12).

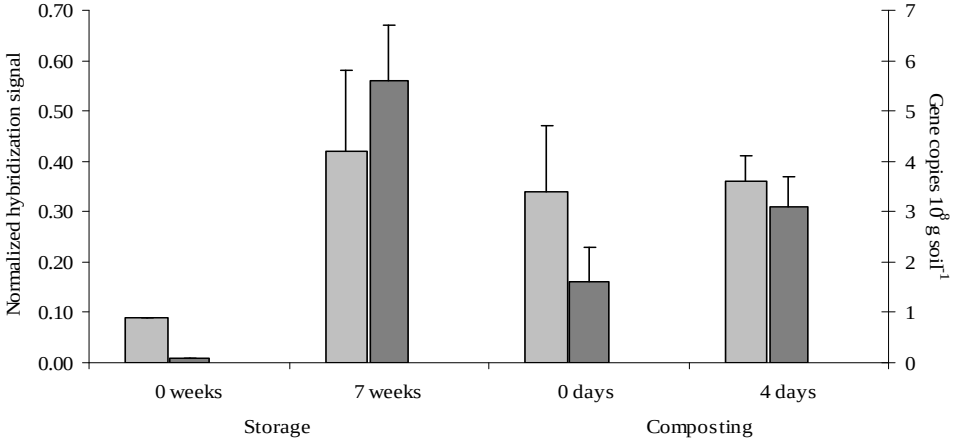

Figure 11. Normalized hybridization signal intensity in the alkane monooxygenase-targeting Mer0307 gene probe (light grey) and the number of alkane monooxygenase gene copies (dark grey) as determined by functional gene microarray and real-time PCR analyses. The analyzed samples were taken after excavation of the diesel oil- and lubrication oil-contaminated soil, after seven weeks of storage in a pile, in the beginning of composting in the BRCSoil bioreactor, and after four days of composting. The error bars represent standard deviations between three replicate DNA isolations.

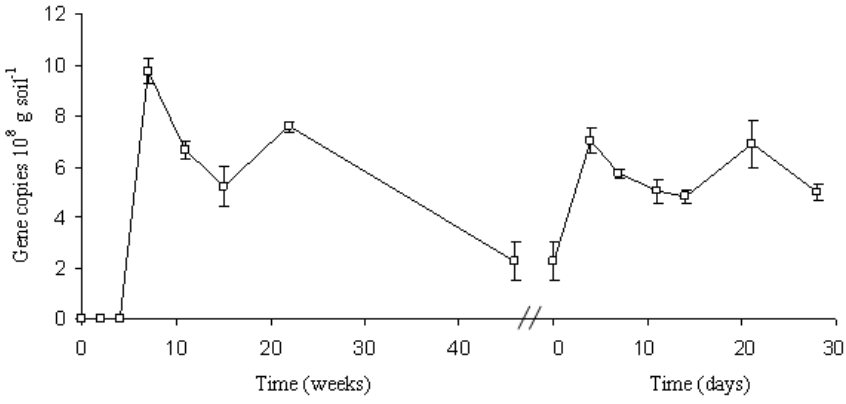

Figure 12. The number of alkane monooxygenase gene copies in the diesel oil- and lubrication oil-contaminated soil during storage in a pile (weeks 0–46) and during composting in the BRCSoil Bioreactor (days 0–28) as determined by dot blot hybridization. The error bars represent standard deviations between three replicate DNA extractions.

The 16S rRNA gene-targeted PCR-DGGE analysis also indicated a similar change in the composition of the bacterial community during storage. After excavation, one 16S rRNA gene fragment with 99 % sequence similarity to uncultured Betaproteobacterium, previously identified from hydrocarbon-contaminated environments, became enriched in the bacterial community profile (Figure 13).

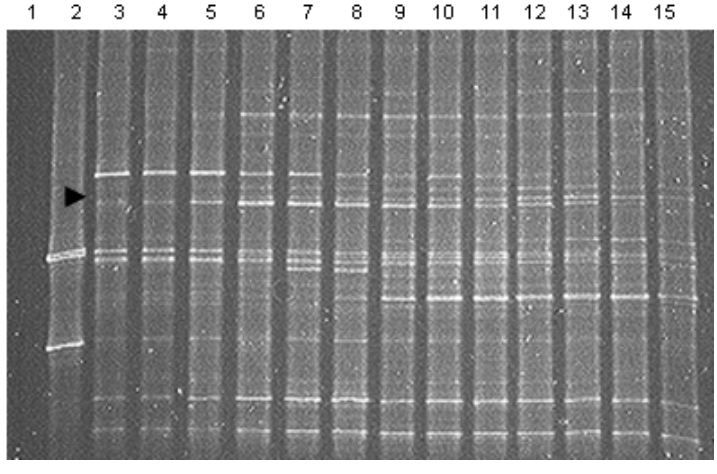

Figure 13. DGGE analysis of 16S rRNA gene fragments PCR amplified from the diesel oil- and lubrication oil-contaminated soil after 0, 2, 4, 7, 11, 22 and 46 weeks of storage in a pile (lanes 3–8), and after 4, 7, 11, 14, 21, 29 days of composting in the BRCSoil Bioreactor (lanes 9–15). Negative and positive controls on lanes 1 and 2. The arrow head indicates the 16S rRNA gene fragment closely similar to the uncultured Betaproteobacterium.

During the next 15 weeks the soil pH and moisture content decreased from 5.7 to 5.2 and 7.1 % to 5.5 %, respectively, thus indicating the development of hypoxic conditions and drying. At the same time the amount of alkane monooxygenase gene also decreased to 7.6 x 10^8 gene copies (Figure 12). A decrease in C_{10}-C_{30} alkane biodegradation following drying was also observed by Kaplan and Kitts (2004) in a land-treatment unit treating petroleum hydrocarbons, amended with nutrients and aerated by tilling. In the end of storage (46 weeks), 2.3 x 10^8 alkane monooxygenase gene copies were detected in one gram of soil and the normalized hybridization signal in the Mer0307 gene probe was 0.34 (±0.13) (Figures 11 and 12). The total hydrocarbon concentration had decreased from an average of 4190 mg kg^{-1} in the beginning of the study to 3370 mg kg^{-1} in the end of storage. The concentration of C_{11}–C_{23} aliphatic hydrocarbons decreased from 3740 mg kg^{-1} to 2700 mg kg^{-1} (29 %), and of C_{24}–C_{39} aliphatic hydrocarbons from 1430 mg kg^{-1} to 640 mg kg^{-1} (55 %).

The molecular biological monitoring data as well as chemical data showed that active hydrocarbon biodegradation was initiated already during storage, after nutrient amendment. This demonstrates that the storage time could be used as an effetive treatment for reducing the time and cost of remediation. While soil excavation and the resulting increase in aeration can, as such, activate n-alkane biodegradation in nutrient-poor soils (Powell et al. 2006), the modifications required for enhancing the long-term efficiency of contaminant-degrading microbes could include regular mixing of the soil mass in conjunction with fertilizer amendment and watering.

The power of molecular biological monitoring methods was especially demonstrated during composting in the BRCsoil bioreactor (Figure 7): The amount of alkane monooxygenase gene copies determined by dot blot hybridization increased to 7 x 10^8 gene copies in one gram of soil during the first four days after nutrient amendment and start of composting (Figure 12). The gene array also indicated a slight increase in the amount of target genes. The normalized hybridization signal intensity in the Mer0307 gene probe increased from 0.34 (±0.13) to 0.36 (±0.05). Together with CO_2 production and FDA hydrolysis activity, this indicated successful reactivation of contaminant biodegradation (Figure 14).

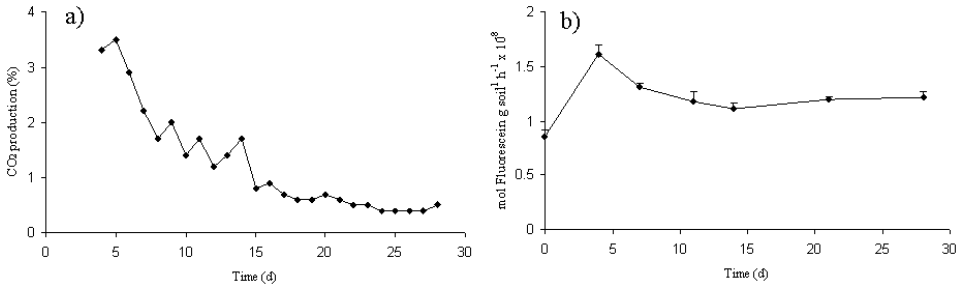

Figure 14. CO_2 production (a) and FDA hydrolysis activity (b) in the diesel oil- and lubrication oil-contaminated soil during composting in the BRCSoil Bioreactor. The highest CO_2 percentage that was measured during the aeration periods was selected to present the CO_2 production of each day. No measurements were obtained during the first four days due to technical problems. The error bars represent standard deviations between three replicate FDA hydrolysis measurements.

However, the increase in the abundance of alkane monooxygenase genes was not as prominent as the increase observed after soil excavation, and no statistically significant difference was observed in hybridization signal intensities in the alkane monooxygenase gene probe during the first four days of composting. Furthermore, the amount of alkane monooxygenase genes and the relative proportion of the Betaproteobacterium in the bacterial community subsequently started to decline, thus illustrating a decrease in the contaminant degradation activity (Figures 12 and 13). The number of alkane-degrading bacteria has been shown to be reduced when the bioavailable substrate is depleted (Powell et al. 2006). It is therefore possible that the decrease in the number of alkane monooxygenase genes was due to the depletion of bioavailable hydrocarbons or to the sequential enrichment of other degraders (Kaplan and Kitts 2004). It is also possible that, in aged soils such as the soil used in this study, the poorly available contaminant fraction may be resistant to degradation even under optimized conditions (Jørgensen et al. 2000). In our study, however, a decrease in CO_2 production and aerobic microbial activity were observed simultaneously with a decline in contaminant-degrading bacteria. This indicated a decrease in the overall activity of the microbial community. Furthermore, after four weeks of treatment in the bioreactor the total hydrocarbon concentration was reduced from 3370 mg kg-1 to 2260 mg kg-1, with 64 % of the C11-C23 and 83 % of the C24-C39 aliphatic hydrocarbons remaining in the soil. Later on, the inefficient decontamination observed early in the process by molecular

biological methods was confirmed by discovery of dislocation of one of the two aeration tubes that resulted in poor aeration during composting (Figure 7).

4.5 Methodological aspects of functional gene array analysis

4.5.1 Sensitivity

One of the challenges in the application of molecular biological methods for monitoring biodegradation in the environment is assay sensitivity. The functional genes often represent only a small fraction of the total community DNA, and the presence of heterogeneous background DNA can reduce the sensitivity of the analysis particularly in hybridization-based applications (Franke-Whittle et al. 2005, Tiquia et al. 2004, Wu et al. 2001).

In this thesis, 250 to 2314 bp and 489 to 620 bp long gene fragments were used as gene probes on the functional gene macro- and microarrays, respectively. The long gene probes were selected because they offer higher detection sensitivity in comparison to the oligonucleotide probes (He et al. 2005, Letowski et al. 2004). With the 489 to 620 bp long probes we detected bacteria representing 1 % of the microbial community or 10^5 copies of the target genes (Table 6, Papers III and IV). Because no prior PCR amplification of target genes was performed, PCR bias that can change the original target abundance and limit the relative comparison of hybridization signal intensities between samples was avoided (Lee et al. 2006, Stalis-Pavese et al. 2004, von Wintzingerode et al. 1997, Wilson 1997).

The detection of 10^5 gene copies corresponds to the current detection limit reported for PCR-amplified gene probes (Cho and Tiedje 2002, Dennis et al. 2003, He et al. 2005). Although the hybridization sensitivity is probe dependent (He et al. 2005, Rhee et al. 2004), the sensitivity of our array is also higher than the sensitivity obtained with oligonucleotide probes that have been reported to detect bacteria representing 5 % of the microbial community or 10^7 gene copies (Franke-Whittle et al. 2005, Rhee et al. 2004, Taroncher-Oldenburg et al. 2003). Nevertheless, in comparison to real-time PCR analysis that detected 10^4 naphthalene dioxygenase and 10^6 alkane monooxygenase gene copies in the field-scale remediation samples and 10^2 copies of quantification standard in one PCR reaction in this thesis and in previous studies (Baldwin et al. 2003, Park

and Crowley 2006, Powell et al. 2006), the current detection level of the gene array analysis, 10^5 gene copies, is clearly insufficient for the detection of low-frequency, but often functionally significant, bacterial populations (Table 6).

Table 6. Summary of the molecular biological methods used in this thesis. Sensitivity indicates the lowest amount of target genes detected in a mixed bacterial culture or in an environmental sample.

Method	Detection	Number of genes analyzed	Substrates	Amount of DNA analyzed	Sensitivity (gene copies)	Specificity (% similarity)	Paper
Dot blot hybridization	^{32}P	1	LMW PAH n-alkanes	500 ng	10^7		I II III
Real-time PCR	SYBR Green I	1	LMW PAH	1 µg 1 µg	10^5 (Soil) 10^4 (Water)	>87	I IV
Functional gene macroarray	^{32}P	7	LMW PAH BTEX	100 ng	9 pg	>80	II
Functional gene microarray	Cy3	16	LMW PAH BTEX n-alkanes	2 µg	10^5	>80	III IV

4.5.2 Specificity

In biodegradation process monitoring, assays that not only target a specific hydrocarbon-degrading genotypes but also provide information on their substrate specificities and biodegradation efficiencies, would be advantageous in predicting the biodegradation potential (Futamata et al. 2001). In paper I, a real-time PCR assay was developed for the enumeration and monitoring of bacteria sharing the same biodegradative function, i.e. the aerobic degradation of LMW PAHs, on the basis of sequence similarities in naphthalene dioxygenase genes of naphthalene-degrading Gamma- and Betaproteobacteria (Table 5). The naphthalene dioxygenase gene fragments PCR amplified from field-scale bioremediation process samples shared over 95 % sequence similarity to the sequences used for primer design, which demonstrated that the real-time assay could be targeted to a common biodegradation function.

In gene array analysis, distinguishing between closely similar, but functionally unrelated, gene sequences is especially important because unspecific cross-hybridization to unknown non-target sequences may lead to over-estimation of the biodegradation potential (Gentry et al. 2006). According to Kane et al.

(2003), continuous, complementary sequence streches of at least 15 base pairs can lead to unspecific hybridization with 50mer oligonucleotide probes. Because the probability of finding these complementary sequences increases along with increasing probe length, long PCR-amplified gene probes used in this thesis are particularly prone to unspecific hybridization. Therefore, highly stringent hybridization conditions were used in all the hybridization analyses throughout this thesis in order to ensure assay specificity (Papers II, III and IV). In addition, in order to ensure uniform hybridization specificities and efficiencies for all the gene probes attached to the functional gene microarray the gene probes were designed to have as similar hybridization characteristics as possible.

The analysis of pure bacterial cultures and a mixed bacterial culture demonstrated that, under the highly stringent conditions, the PCR-amplified gene probes specifically detected genes sharing less than 80 % sequence similarity and a common, conserved biodegradation function, while discriminating between genes involved in the degradation of aliphatic, monoaromatic and polyaromatic hydrocarbons (Table 6, Figure 2 in paper II, Figure 1 in paper III and Table S3 in paper IV). Closely similar genes were also specifically detected in soil slurry microcosm and creosote-contaminated groundwater (Papers III and IV). In the soil slurry microcosm, the naphthalene dioxygenase and catechol dioxygenase genes detected by the functional gene microarray shared 95 % and 96 % sequence similarity with the respective gene probes, respectively. Moreover, as a result of probe optimization, consistent hybridization signals were detected in the gene probes targeting different genes on the naphthalene biodegradation pathway (Paper III).

The results agree with previous reports showing that, with long gene probes, gene sequences sharing 79 to 85 % sequence similarity can be differentiated (Steward et al. 2004, Wu et al. 2001, Table 2). Although this is slightly lower than the 87-94 % sequence similarity differentiated with 50mer and 70mer oligonucleotide probes (Rhee et al. 2004, Taroncher and Oldenburg et al. 2003, Tiquia et al. 2004), it is more advantageous in bioremediation process monitoring to identify a conserved and relevant biodegradation function responsible for the degradation of a specific contaminant than an individual genotype (Watanabe et al. 2002).

However, it is not possible to obtain one set of hybridization conditions optimally specific for all probes (Letowski et al. 2004). One way to avoid both non-specific and false-negative hybridization signals would be to use multiple probes for the same target gene (Franke-Whittle et al. 2008, He et al. 2007,

Peplies et al. 2006, Sanguin et al. 2006). In this thesis, the design of gene probes for key degradation enzymes on both the upper and lower part of each biodegradation pathway increased the confidence of the results and allowed the simultaneous detection of different parts of naphthalene and toluene biodegradation pathways in a mixed bacterial culture (Figure 2 in paper II) and in a soil slurry microcosm (Figure 4 in paper III). The detection of biodegradation pathways would be especially important in monitoring bioremediation processes where complete removal of contaminants is necessary in order to ensure that the remediation goals are achieved successfully.

4.5.3 Coverage

Even the most specific and sensitive analysis can only be used to detect biodegradation functions that it was designed to target. In this thesis, the real-time PCR analysis and the small-scale functional gene arrays were used for method development and validation. It is clear that the targeted genes do not represent the genetic diversity of contaminant-degrading bacteria in the environment. In the diesel oil- and lubrication oil-contaminated soil, for instance, the alkane monooxygenase gene was only detected with a gene probe that was prepared from the investigated soil samples. New primers and gene probes are therefore needed to target more diverse biodegradation functions. These include, for example, the PAH-degradative *nar*-genes from Gram positive *Rhodococcus* strains (Larkin et al. 1999) and the *nid*-genes from HMW PAH-degrading *Mycobacterium* species (Kim et al. 2007). Because the available sequence data contains mainly gene sequences closely similar to the culturable fraction of environmental diversity, which in fact often does not represent the genetic diversity in the environment (Windler et al. 2007), it is necessary to screen for novel genes that could be used as gene probes also from contaminated environments. In this thesis the focus was set on aerobic biodegradation because the engineered treatment techniques often use aerobic treatment, and aerobic biodegradation is also thermodynamically more feasible (Madigan et al. 2003). However, anaerobic biodegradation of contaminants can also be a significant process under the anoxic conditions that often develop in polluted subsurfaces (Anderson and Lovley 1997). Therefore, probes that target genes involved in anaerobic hydrocarbon biodegradation and different terminal electron accepting processes may also be advantageous in the remediation process monitoring. This

was demonstrated by the detection of the sulphide reductase (*dsr*A) gene in the creosote-contaminated groundwater (Paper IV).

4.5.4 Quantification

Direct, quantitive comparison between numerous time points is essential for understanding the microbial dynamics in the monitoring of biodegradation processes. In real-time PCR analysis, the target gene was quantified using quantification standards. High precision with less than 3 % intra-assay variability was observed (Paper I). This is similar to 3–5 % variation between replicate samples observed in previous studies (Smith et al. 2005).

The hybridization signal intensities obtained with the preliminary functional gene macroarray during naphthalene and toluene biodegradation in the mixed bacterial culture were also in good agreement with the amount of naphthalene and toluene dioxygenase genes determined by dot blot hybridization (Figure 4 in paper II). Consistent with other reports showing good agreement between hybridization signal intensities and the amount of target DNA this suggests that, at least within a certain range, gene array analysis could potentially also be used at least for the semi-quantitative assessment of target genes in environmental samples (Rhee et al. 2004, Steward et al. 2004, Tiquia et al. 2004, Yin et al. 2007, Wu et al. 2001).

However, due to the inherent variation associated with hybridization analysis, the signal intensities between multiple samples cannot be directly compared (Franke-Whittle et al. 2005). In order to allow the direct comparison between multiple samples, we therefore combined the functional gene microarray analysis with hybridization signal normalization using internal standard probes. Because hybridization signal normalization based on standard probes does not require reference samples, the normalized signal intensities can be directly compared across several time points and treatments or even used to quantitate the target abundance (Carter et al. 2005, Dudley et al. 2002). Internal standards are useful in controlling for hybridization variability also in environmental array applications where the differences in target gene abundance have commonly been compared using reference samples and competitive two colour hyridization (Cho and Tiedje 2002, Lievens et al. 2005, Moisander et al. 2006, Peplies et al. 2006).

In this thesis, adding the internal standard probes to the sample DNA prior to labelling was effective in increasing hybridization reproducibility in all the samples analysed. As a result, smaller differences in the relative amount of

target genes were discriminated and a linear relationship between the amount of target genes and normalized hybridization signal intensities was observed down to 20 ng of genomic DNA in the presence of heterogeneous background DNA (Figure 2 and Table 3 in paper III). In the soil slurry microcosm the functional gene microarray results correlated with the amount of naphthalene dioxygenase and catechol dioxygenase gene copies determined by dot blot hybridization (Paper III). Good agreement was also observed between the hybridization signal intensities and the abundance of target genes determined by real-time PCR in the environmental samples recovered from both *in situ* and *ex situ* bioremediation processes (Paper IV).

However, no statistically significant differences were detected between the two time points analyzed from the soil slurry microcosm or the composting process of the diesel- and lubrication oil-contaminated soil. Also, in the mixed bacterial culture, correlation was observed between the functional gene array analysis and the amount of target genes only after three biological replicates had been analyzed (Paper II). High hybridization signal variation between replicate samples was also reported in marine microbial mat samples and uranium-contaminated sediment samples by Moisander et al. (2006) and Chandler et al. (2006), which indicates that, in order to acquire environmentally significant data and reliably interpret microbial community dynamics during environmental processes, adequate replication using true biological replicates is required (He et al. 2007). Replication and sampling are especially important in environments in which there is significant spatial heterogeneity in the presence of microorganism, or where different physiological zones are present in close proximity (Bekins et al. 1999). For example, in Paper IV the simultaneous detection of the sulphide reductase gene and aerobic PAH-degradation genes suggest that mixing of the different redox zones may have occurred during sampling.

The detection of small differences in the abundance of target genes may also be limited by the low dynamic range of functional gene array analysis. In both the soil slurry microcosm and the *ex situ* remediation of the diesel- and lubrication oil-contaminated soil, the real-time PCR analysis showed statistically significant differences between the analyzed samples. Even though the hybridization signal intensities in the functional gene probes also agreed with real-time PCR, statistically significant differences between the respective samples could not be determined with the functional gene microarray (Paper III). The dynamic range of gene array analysis has commonly been evaluated to be three, or in some cases even five, orders of magnitude (Brodie et al. 2007,

Lievens et al. 2005, Neufeld et al. 2006, Rhee et al. 2004, Wu et al. 2001), while the dynamic range of real-time PCR assay can be seven to eight orders of magnitude (Lee et al. 2006, Powell et al. 2006).

The quantitativity is also strongly dependent on the probe specificity (Gentry et al. 2006). Although the functional gene array proved successful in monitoring specific degradative populations over time, the quantification may become problematic if different genotypes conferring to the same biodegradation function but having different hybridization efficiencies or different gene copy numbers become enriched during degradation (Chandler et al. 2006, Lee et al. 2006, Stralis-Pavese et al. 2004). In addition, it is necessary to remember that it is not possible, due to the differencies in hybridization efficiencies, to quantitatively compare hybridization signals between probes from the same hybridization (Chandler et al. 2006, Franke-Whittle et al. 2005).

5. Conclusions

The abundance of functional genes involved in hydrocarbon biodegradation can be used as an indicator of the hydrocarbon biodegradation potential and efficiency. By investigating the functional genes with methods such as quantitative real-time PCR, dot blot hybridization and functional gene array analysis, it is possible to monitor the succession of hydrocarbon-degrading bacteria during hydrocarbon biodegradation and to evaluate the feasibility of different treatments for enhancing hydrocarbon biodegradability in both laboratory- and field-scale applications. With careful assay design, the analysis can be targeted at relevant biodegradation functions instead of specific genotypes. However, straightforward interpretation of contaminant concentrations based on gene copy numbers should be avoided.

In terms of assay feasibility, the real-time PCR quantification of functional genes is clearly the most sensitive method for studying individual genes in environmental samples, whereas dot blot hybridization forms a robust method for simultaneous analysis of several environmental samples. The parallel detection of different biodegradative genes by functional gene array hydridization represents an especially potential tool for investigating hydrocarbon biodegradability in the environment:

- The hybridization signal intensities obtained by the functional gene arrays were in good agreement with the mineralization of specific hydrocarbons, the abundance of hydrocarbon-degradative genes and overall changes in the microbial community composition.
- The biodegradation of several different hydrocarbons could be monitored simultaneously.
- Both lower and upper pathway genes required for degradation of hydrocarbons could be detected simultaneously.

- The hybridization results supported the physico-chemical and microbiological monitoring data obtained during field-scale remediation processes.
- Genes involved in the biodegradation of different hydrocarbons and genes sharing less than 80 % sequence similarity were specifically detected.
- Hydrocarbon-degrading bacteria representing 1 % of the total microbial community could be detected without prior PCR amplification of the target genes. Using gene fragments as gene probes increased the sensitivity of the functional gene array analysis in comparison to published oligonucleotide arrays.
- Multiple samples and hybridization analyses were successfully compared when the hybridization signal intensities were normalized using internal standard probes.

6. Future outlook

The analysis of hydrocarbon-biodegradative genes provides a powerful tool for increasing our understanding of the role of contaminant-degrading bacterial dynamics in bioremediation processes and for developing more efficient treatment processes. However, all the methods have their pros and cons: In environments contaminated with complex mixtures of hydrocarbons, parallel analysis of numerous functional genes by gene array analysis allows a more comprehensive investigation of the required biodegradation potential. At the present time, however, the relatively low sensitivity, the challenges in assay quantitativity and specificity, as well as our limited knowledge of the microbial diversity in the environment, may limit its routine applications in the monitoring of full-scale bioremediation processes. Real-time PCR, on the other hand, provides a more sensitive and quantitative assay for measuring the abundance of specific biodegradation functions. Although the obtained numerical data may be more valuable in decision making and process management, only a small number of activities can be simultaneously investigated. In the future, many of the technical challenges as well as the need for additional gene sequence data are expected to be answered by advances in analysis techniques and large scale genomic and metagenomcic sequencing efforts. However, even then, one must always consider what are the questions that need to be answered in the specific study, and what kind of method would most feasibly and reliably provide the required information before selecting any particular method.

In this study, we used DNA samples for hybridization and PCR analyses. Although this proved valuable in monitoring the dynamics of hydrocarbon-degrading bacteria, the presence of a specific gene sequence is not necessarily indicative of its activity. In order to link the functionality to biodegradation activity, analysis of messenger RNA is required. Additional gene probes targeting more diverse functional genes are needed to better cover the metabolic

and genetic diversity of hydrocarbon-degrading microbes in the environment. More comprehensive standardization of analysis methods is also required. This includes normalization of hybridization signal intensities with additional standard probes and, regardless of the analysis method, standardization for differences in DNA isolation efficiency. It is also necessary to improve the dynamic range of gene array analysis which, at its current level, seems to limit the reliable detection of small differences in target gene abundance.

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Mari Nyyssönen

Title

Functional genes and gene array analysis as tools for monitoring hydrocarbon biodegradation

Abstract

Bioremediation is considered to be an environmentally sustainable and cost-effective means for environmental clean-up. However, a comprehensive understanding of the biodegradation potential of intrinsic microbial communities and their response to decontamination measures is required for the effective management of bioremediation processes.

In this thesis, the potential to use functional genes encoding hydrocarbon-degradative enzymes as indicators of aerobic hydrocarbon biodegradation was investigated. Small-scale functional gene macro- and microarrays targeting aliphatic, monoaromatic and low molecular weight polyaromatic hydrocarbon (PAH) biodegradation were developed in order to simultaneously monitor the biodegradation of mixtures of hydrocarbons. The validity of the array analysis in monitoring hydrocarbon biodegradation was evaluated in microcosm studies and field-scale bioremediation processes by comparing the hybridization signal intensities to hydrocarbon mineralization, real-time polymerase chain reaction (PCR), dot blot hybridization and both chemical and microbiological monitoring data.

The results obtained by real-time PCR, dot blot quantification and gene array analysis were in good agreement with hydrocarbon biodegradation. In laboratory-scale microcosms, mineralization of several hydrocarbons could be monitored simultaneously using functional gene array analysis. In the field-scale bioremediation processes, the detection and enumeration of hydrocarbon-degradative genes provided important additional information for process optimization and design. In creosote-contaminated groundwater, the functional gene array analysis demonstrated that the aerobic PAH-biodegradation potential that was present at the site, but restrained under the oxygen-limited conditions, could be successfully stimulated with air sparging and nutrient infiltration. During *ex situ* bioremediation of diesel oil- and lubrication oil-contaminated soil, the functional gene array analysis revealed inefficient hydrocarbon biodegradation, caused by poor aeration during composting. The functional gene array specifically detected upper and lower biodegradation pathways required for complete mineralization of hydrocarbons. Hydrocarbon-degrading bacteria representing 1 % of the microbial community could be detected without prior PCR amplification.

Molecular biological monitoring methods based on functional genes provide powerful tools for the development of more efficient remediation processes. With careful assay design, the analysis can be directed to common biodegradation functions rather than specific genotypes. The parallel detection of several functional genes using functional gene array analysis is an especially promising tool for monitoring the biodegradation of mixtures of hydrocarbons.

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Mari Nyyssönen

Nimeke

Toiminnalliset geenit ja geeniarray-analyysi hiilivetyjen biohajoamisen seurantatyökaluina

Tiivistelmä

Biopuhdistuksessa pilaantunutta ympäristöä kunnostetaan ympäristössä luonnollisesti esiintyviä, haitallisia yhdisteitä hajottavia mikrobeja hyväksi käyttäen. Sitä pidetään ympäristön kannalta kestävänä sekä taloudellisesti kannattavana kunnostusmenetelmänä. Biologisten puhdistusprosessien hallitseminen vaatii kuitenkin hyvää ymmärrystä luontaisten mikrobiyhteisöjen hajotuspotentiaalista sekä niissä kunnostuksen seurauksena tapahtuvista muutoksista.

Väitöskirjatutkimuksessa tutkittiin hiilivetyjen hajotuksesta vastaavia entsyymejä koodaavien toiminnallisten geenien soveltuvuutta hiilivetyjen aerobisen biohajoamisen seurantaan. Jotta useiden hiilivetyjen biohajoamista voitaisiin tutkia samanaikaisesti, tutkimuksessa kehitettiin suoraketjuisten sekä monoaromaattisten ja pienimolekyylisten polyaromaattisten hiilivetyjen (PAH) hajotusgeeneistä koostuvat pienimuotoiset makro- ja mikroarrayt. Menetelmien toimivuutta hiilivetyjen biohajoamisen seurannassa arvioitiin sekä laboratoriomittakaavan mikrokosmoskokeissa että kenttämittakaavan biopuhdistusprosesseissa vertaamalla arraytuloksia hiilivetyjen mineralisaatioon, reaaliaikaiseen polymeraasiketjureaktioon (PCR), dot blot -hybridisaatioon sekä kemiallisilla ja mikrobiologisilla seurantamenetelmillä saatuihin tuloksiin.

Reaaliaikaisella PCR:llä sekä funktionaalisella geeniarraylla mitattu toiminnallisten geenien määrä oli hyvässä yhteydessä hiilivetyjen mineralisaatioon. Geeniarrayn avulla voitiin tutkia useiden hiilivetyjen biohajoamista samanaikaisesti laboratoriomittakaavan mikrokosmoskokeissa. Hajotusgeenien tutkiminen molekyylibiologisten menetelmien avulla lisäsi biologisten kunnostusprosessien optimoinnissa ja suunnittelussa tarvittavaa tietoa myös kenttämittakaavan biopuhdistusprosesseissa. Kreosootilla pilaantuneessa pohjavedessä funktionaalinen geeniarray osoitti, että pilot-mittakaavan biologisesti reaktiivisella seinämällä voitiin aktivoida kohteessa esiintyvää, mutta hapettomien olosuhteiden rajoittamaa, hajotuspotentiaalia. Diesel- ja voiteluöljyllä pilaantuneen maan ex situ -kunnostuksen aikana funktionaalinen geeniarray puolestaan paljasti jo prosessin aikaisessa vaiheessa puutteellisen ilmastuksen aiheuttaman biohajoamistehokkuuden heikentymisen. Geeniarray tunnisti spesifisesti hiilivetyjen täydelliseen mineralisaatioon tarvittavat kokonaiset hajotusreitit. Hiilivetyjä hajottavat bakteerit oli mahdollista tunnistaa ilman hajotusgeenien PCR-monistusta, kun bakteerit edustivat yhtä prosenttia koko mikrobiyhteisöstä.

Tulokset osoittavat, että toiminnallisiin geeneihin perustuvat molekyylibiologiset seurantamenetelmät muodostavat tehokkaita työkaluja, joiden avulla voimme kehittää entistä tehokkaampia kunnostusprosesseja. Hyvällä suunnittelulla analyysi voidaan kohdentaa yksittäisen bakteerien tunnistamisen sijasta keskeisiin hajotusaktiivisuuksiin. Useiden hajotusgeenien samanaikainen tunnistaminen geeniarrayhybridisaatioon perustuen on erityisen lupaava menetelmä hiilivetyseosten biohajoamisen seurantaan.

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