Biodegradation (2005) 16: 127–134

Monitoring of accelerated naphthalene-biodegradation in a bioaugmented soil slurry

Reetta Piskonen*, Mari Nyyssönen, Tiina Rajamäki & Merja Itävaara
*VTT Biotechnology, P.O. Box 1500, 02044 VTT, Finland (*author for correspondence: e-mail: reetta.piskonen@vtt.fi)*

Key words: bioremediation, inoculation, molecular monitoring, naphthalene, soil slurry

Abstract

The effect of microbial inoculation on the mineralization of naphthalene in a bioslurry treatment was evaluated in soil slurry microcosms. Inoculation by *Pseudomonas putida* G7 carrying the naphthalene dioxygenase (*nah*A) gene resulted in rapid mineralization of naphthalene, whereas indigenous microorganisms in the PAH-contaminated soil required a 28 h adaptation period before significant mineralization occurred. The number of *nah*A-like gene copies increased in both the inoculated and non-inoculated soil as mineralization proceeded, indicating selection towards naphthalene dioxygenase producing bacteria in the microbial community. In addition, 16S rRNA analysis by denaturing gradient gel electrophoresis (DGGE) analysis showed that significant selection occurred in the microbial community as a result of biodegradation. However, the indigenous soil bacteria were not able to compete with the *P. putida* G7 inoculum adapted to naphthalene biodegradation, even though the soil microbial community slightly suppressed naphthalene mineralization by *P. putida* G7.

Introduction

Slurry phase conditions have been successfully applied in the bioremediation of petroleum hydrocarbon and aromatic hydrocarbon contaminated soils (DiGrazia et al. 1991; Hwang & Cutright 2002; Nano et al. 2003; Ramirez et al. 2001). Monitoring bioreactor-type remediation systems is typically based on the determination of pollutant concentration (Nano et al. 2003). However, an extensive understanding of microbiological processes is of considerable benefit when optimizing biodegradation processes. In bioreactors with pure cultures, the determination of microbial numbers by plate counts can be used to evaluate reactor performance. However, in soil slurry reactors the soil itself normally contains a rich microbial population, of which only a fraction can be detected by the traditional cultivation methods (Watanabe & Baker 2000). Furthermore, methods that describe the general activity of microbial communities may not adequately describe the activity of the bacteria participating in the degradation processes. For instance, Margesin et al. (2003) found no correlation between general microbiological parameters such as respiration enzyme activities and the hydrocarbon degradative genotypes. Recent developments in molecular microbiology have increased the possibilities to analyze changes at the community level without cultivation or other indirect determinations (Cavalca et al. 2002; Hallier-Soulier et al. 1996; Head et al. 1998; Muyzer et al. 1993; Sanseverino et al. 1993; Watanabe & Baker 2000). These methods, which are based on the direct analysis of DNA extracts, can clarify several of the phenomena occurring in the biodegradation process (Plaza et al. 2001).

According to Sanseverino et al. (1993), the ability of soil microorganisms to biodegrade hydrocarbons can be estimated by analyzing the catabolic genes present in soil. Enrichment of catabolic gene-carrying bacteria seems to be a common phenomenon during the course of biodegradation. Whyte et al. (1999) observed that

the proportion of *alkB*- genes in the cultivable consortium increased when hexadecane mineralization started. Guo et al. (1997) showed that catabolic genes became enriched over the course of time in contaminated soil; however, they did not determine the decrease in contaminant concentrations during the study. Layton et al. (1994) reported increased *bph*-gene numbers during PCB degradation in soil. Ringelberg et al. (2001) reported a similar finding for aromatic hydrocarbon degrading populations. These results demonstrate that catabolic gene based analysis can be a valuable tool for monitoring the efficiency of the degradation processes. However, in order to apply gene-based methods for monitoring the efficiency of the bioremediation processes, suitability of gene assays for quantitative monitoring of the biodegradation processes from the beginning until the end needs still to be clarified.

In this study, the number of large subunits of naphthalene dioxygenase-encoding *nahA*-genes was compared to the mineralization of naphthalene, which acted as a model compound for PAHs in a soil slurry microcosm. The ability of the gene-based methods to determine the endpoint of biodegradation was investigated by taking the final samples after mineralization had ceased. In bioreactors, the time required to reach the remediation goal is directly related to the remediation costs and shortened treatment times are therefore favourable. Inoculation of soil slurries with contaminant-degrading bacteria may result in an enhanced degradation rate, as observed by Wenk et al. (1998) in atrazine-contaminated soil and by Whyte et al. (1999) in petroleum hydrocarbon contaminated soil. Therefore, we evaluated the biodegradation enhancing effect of inoculation with naphthalene-degrading *Pseudomonas putida* G7 by comparing the bioaugmented soil slurry to non-inoculated one. The structure of the microbial community was also analyzed in order to monitor the survival of the inoculum and to relate the gene numbers to microbial succession.

Materials and methods

All chemicals were obtained from Biorad Laboratories and Sigma Aldrich Chemical Co. unless otherwise stated.

Bacterial strains and growth conditions: *Pseudomonas putida* G7 (DSM 6899) carrying the NAH7 plasmid for degradation of naphthalene (Simon et al. 1993) was grown on DSMZ mineral medium 457. *Pseudomonas putida* (DSM 291), *Escherichia coli* (DSM 30083), were grown on Nutrient Broth (Oxoid, UK), and *Bacillus subtilis* (DSM10) on Nutrient Agar (Difco, USA).

Soil was excavated from a PAH-contaminated site and restored in a pile at the Ekokem hazardous waste treatment plant (Finland). Two kilogram of soil was sampled from the pile and passed through a 5 mm sieve. The dry weight was determined by drying overnight at 105 °C. The ash content of the soil was determined after combustion of dry soil at 550 °C for 15 h. The pH of the soil was determined according to Foster (1995) and measured with an Orion model 720 pH meter (Orion Research Inc., USA). PAHs were extracted from soil with toluene. About 0.04 g of the sample was weighted in the Erlenmeyer flask, and d_{10} pyrene, β,β-binaphthyl and indeno[1,2,3-cd] fluoranthene were added as internal standards. Extraction with 50 ml of toluene was performed in the ultrasonic bath (Branson 3210, USA) for 5 min followed by filtration through filter paper (Schleicher & Schuell 589 Black Ribon, Germany). About 1 ml of the filtrate was mixed with 9 ml of hexane. In order to purify the mixture, it was transferred to separation funnel and extracted with 10 ml of dimethylsulphoxide (DMSO). The funnel was shaken and phases were allowed to form for 5 min. DMSO phase formed in the bottom of the funnel was removed and the extraction was repeated twice. Three DMSO extracts were pooled and 60 ml of deionized water was added. DMSO–water mixture was extracted three times with 30 ml of hexane. Hexane extracts were pooled and washed twice with 10 ml of deionized water. DMSO residues were removed by filtrating the extract through a funnel filled with Na_2SO_4. Three millilitres of the extract was further purified with a silica column (J.T. Baker, USA) prewashed with hexane. The sample was eluted from the silica column with 30 ml of hexane and concentrated to 2 ml with rotavapor (Heidolph WB 2000, Germany, Vacuubrand MZ2C Diaphragm vacuum pump, Germany). Further concentration to 1 ml was performed with nitrogen gas (AGA, N_2 5.0, Finland) in 2-ml GC vial.

The concentration of PAHs was analyzed on an HP 5890 gas chromatograph equipped with a DB17 column (J&W Scientific, USA) and HP 5870 mass detector (Hewlett Packard, USA). The temperature of the injector and the detector was 250 °C, and the temperature program was: 80 °C for 12 s, ramp at 35 °C min^{-1} to 160 °C followed by 3 °C min^{-1} to 300 °C and hold for 16 min. The soil properties are given in Table 1.

Table 1. Properties of PAH-contaminated soil

Soil properties	
pH (soil)	6.4
pH (soil suspension)	7.0
Dry weight (%)	87.7 ± 0.8%
Ash content (%)	79.5 ± 0.2%
Total PAHs (mg kg^{-1})	21.0
Naphthalene	0.39
1-methylnaphthalene	0.14
2-methylnaphthalene	0.25
Biphenyl	0.08
Acenaphthylene	0.05
Acenaphthene	0.54
Fluorene	0.31
3-methylbiphenyl	0.03
Dibentsofurane	0.27
Dibentsotiophene	0.09
Phenanthrene	1.5
Anthracene	0.20
2-methylanthracene	0.27
1-methylanthracene	0.09
2-phenylnaphthalene	0.12
Fluoranthene	5.5
Pyrene	3.8
Benzo(a)fluorene	0.40
Benzo(b)fluorene	0.26
Benz(b)naphtho(2,1d)-tiophene	0.39
Benz(a)naphtho(1,2d) tiophene	0.05
Benzo(a)anthracene	0.81
Kryseeni/Triphenylene	1.08
Benzo(b)fluoranthene	1.1
Benzo(k)fluoranthene	0.39
Benzo(e)pyrene	0.9
Benzo(a)pyrene	0.87
Perylene	0.25
Indeno(1,2,3-cd)pyrene	0.50
Benzo(g,h,i)perylene	0.42
Dibenzo(a,h)anthracene	0.06
Coronene	0.06

The mineralization of naphthalene and the efficiency of microbial inoculation were studied in a mineral medium (DSMZ medium 457) containing 10% of PAH-contaminated soil. Six millilitres of the soil slurry was transferred into a 30-ml-glass vial (Applied Sensor, Sweden) containing a 4 ml vial (Chromacol, UK) with 0.5 ml of NaOH as a trap for evolved CO_2. Naphthalene was dissolved in acetone and added to the medium to give a final concentration of 30 mg l^{-1}. *Pseudomonas putida* G7 (DSMZ 4476) carrying the naphthalene dioxygenase (*nah*A) gene was pre-grown in the mineral medium to the log phase and then diluted to 0.05 of A_{600}, after which 150 μl was inoculated into the soil slurry. The mineralization-enhancing effect was compared to non-inoculated soil slurry. The ability of the indigenous microbes to inhibit the growth of *P. putida* G7 was evaluated in autoclaved soil slurry inoculated with the *P. putida* G7-strain. Abiotic controls were treated with 0.5 ml of 4N H_2SO_4. ^{14}C-naphthalene (180,000 dpm) dissolved in acetone was added to the vial just before the bottles were closed with a screw cap containing a Teflon-lined septum (Applied Sensor, Sweden). The microcosms were incubated in the dark at + 22 °C for 7 days.

Naphthalene biodegradation in the different treatments was monitored by determining the amount of $^{14}CO_2$ evolved. For $^{14}CO_2$ determination, 4 replicate microcosms and 3 abiotic controls were sacrificed at each sampling time. Sampling of the *P. putida* G7-inoculated microcosms was performed at 0, 6, 9, 12, 24, 96, 168 h, and the non-inoculated soil slurry was sampled at 0, 18, 24, 28, 36, 44, 96, 168 h. The 0.5 ml NaOH trap was mixed with 4 ml of Wallac OptiPhase HiSafe 3 scintillation cocktail (Wallac, Finland) and the radioactivity was determined on a Wallac 1410 liquid scintillation counter (PerkinElmer, USA). Activity in the abiotic microcosm was subtracted from that in the biotic microcosm, and the biodegradation percentage was calculated by dividing this value by the activity of the ^{14}C-naphthalene added to the vial.

For determination of naphthalene concentration in the soil slurry at the end of the study, 120 ml serum bottles (Sun International Trading, USA) containing 24 ml soil slurry were run under the same conditions as in the 30-ml vials. However, no radioactively labelled naphthalene was

added to the bottles. Naphthalene concentration in the slurry was determined by gas chromatography using the static headspace technique with a Tekmar 7000 headspace autosampler (Tekmar Dohrmann™, USA) and HP 5890 Series II gas chromatograph (Hewlett Packard, USA) equipped with an HP-5 column (50 m, 0.2 mm i.d., film thickness 0.33 μm, supplied by Hewlett Packard, USA) and flame ionization detector (FID). The samples were equilibrated at 80 °C for 30 min. The temperature of the injector was 150 °C. The separation was performed with the following temperature program: 60 °C, for 2 min, ramp at 5 °C min^{-1} to 100 °C followed by ramp at 20 °C min^{-1} to 250 °C and hold for 5.5 min. The temperature of the detector was 250 °C. Helium was used as carrier gas at a pressure of 200 kPa. Samples and standards were prepared in headspace vials (volume 22 ml) that were sealed with PTFE/Butyl-septa and aluminium caps (CRS, USA).

For the DNA analysis of the microbial community, the 30-ml microcosms were prepared as previously, but radioactively labelled naphthalene was not added to the vial. Six replicates were sacrificed at each sampling time. Medium was transferred into 50-ml plastic tubes (Corning, USA) and centrifuged (4000 rpm, 5 min). DNA was extracted according to Stephen et al. (1999). The 193 bp fragment of 16S rDNA was amplified by P2 and P3 primers (Muyzer et al. 1993). The PCR products were separated by denaturing gradient gel electrophoresis (DGGE) on 8% acrylamide with an increasing denaturant gradient from 40 to 60%. The 100% denaturant was composed of 7 M urea and 40% formamide. Electrophoresis was run on the Decode universal mutation detection system (Biorad, USA) in 0.5 × TAE buffer (20 mM Tris acetate, 0.5 mM EDTA, pH 8) at 60 °C for 18 h according to Muyzer et al. (1993). After staining the gels with SybrGreen I (BMA, Denmark) they were analyzed on a Gel Doc 2000 UV transilluminator (Biorad, USA). The dominant bands were cut from the gel. The DNA was diluted in water and reamplified with the P2 and P3 primers as described earlier, followed by purification with the Qiaquick PCR purification kit (Qiagen, Germany). Amplicon was prepared for sequencing with the ABI Prism BigDye Terminator v3.0 sequencing standard kit (PE Applied Biosystems, UK), and sequenced with the ABI Prism 310 genetic analyzer (Applied Biosystems, USA). Sequences were compared to the GenBank sequences using the BLAST search (Altschul et al. 1997).

For catabolic gene analysis, the plasmid DNA was extracted from *P. putida* G7 according to Sambrook & Russel (2001). The 866 bp fragment of the *nah*A gene was amplified from plasmid DNA according to Ferrero et al. (2002). The *nah*A gene probe was generated by PCR using the same procedure, except that 50 μCi of [α-^{32}P dCTP]. (Amersham Biosciences, UK) was added to the reaction mixture in order to generate radioactively labelled probe. The probe was purified with the Qiaquick PCR purification kit and its activity was determined on the Wallac 1410 liquid scintillation counter.

In order to determine the amounts of *nah*A-like genes by means of hybridization, 0.5 μg total DNA extracted from the soil slurry, 1 μg total DNA from the *P. putida G7* as positive control, and 1 μg total DNA from *B. subtilis*, *P. putida* and *E. coli* as negative controls, were denatured in 0.45 M NaOH for 10 min at room temperature and then filtered on separated locations on a Hybond-N nylon membrane (Amersham Biosciences, UK) with the Bio-Dot filter unit (Biorad, USA). The wells were washed with 2 × SSC (1 × SSC contains 0.15 M NaCl and 0.015 M sodium citrate). For quantification of the genes in the DNA samples, an 866 bp fragment of the *nah*A gene at different concentrations (0.03–10 ng) was attached on the membrane. All the samples, controls and standards were blotted in three replicates. The membrane was cross-linked with the UV Stratalinker 2400 (Stratagene, USA).

Hybridization was performed in highly stringent conditions according to Sanseverino et al. (1993) with the following buffer: 0.5 M $NaH_2PO_4 \cdot \times H_2O$, 1 mM EDTA, 7% SDS, pH 7.2. The membrane was prehybridized in 15 ml of buffer for 90 min at 68 °C. The *nah*A-probe was denatured at 95 °C for 10 min. The buffer was changed and 500,000 dpm ml^{-1} of the probe was added. Hybridization was run at 68 °C for 18 h in an hybridization oven (Hybaid, UK). After hybridization, the membrane was rinsed for 30 min with 2 × SSC containing 0.1% SDS, followed by washing with 1 × SSC containing 0.1% SDS and finally with the stringent washing buffer (10 mM NaCl, 20 mM Tris Base, 1 mM EDTA, 0.5% SDS, pH 7–8). The membrane was exposed on the

phosphor screens for 3 h (Molecular Probes, USA), and the screen was scanned by a Typhoon 8600 phosphoimager (Molecular Probes, USA). The intensity of the spots developed on the screens was quantified with the ImageQuant program (Molecular Probes, USA).

Results and discussion

Inoculation of *P. putida* G7 into the soil slurry resulted in rapid mineralization of naphthalene: in 6 h 20% and 36% of the ^{14}C-naphtahalene was converted into CO_2 in the *P. putida* G7 amended soil slurry and in the autoclaved soil slurry amended with *P. putida* G7, respectively (Figure 1). The difference was statistically significant (*t*-test, $p < 0.01$), which indicates that the activity of *P. putida* G7 was suppressed by the indigenous microbial community at the beginning of the study. However, after 9 h the difference between the treatments was no longer statistically significant. Furthermore, the mineralization in both treatments had reached the plateau phase, where 60% of the naphthalene had been mineralized, after 24 h. In the non-inoculated soil slurry, a significant lag period of 28 h was observed before the onset of mineralization (Figure 1). After the lag period, the mineralization of naphthalene occurred rapidly but slowed down after 44 h when 60% of the naphthalene had been converted into CO_2. According to chemical analysis, there was no naphthalene left in the medium at the end of the 7-day study.

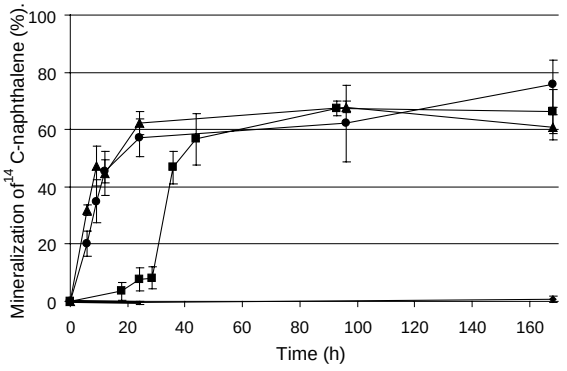

Figure 1. Mineralization of ^{14}C-naphthalene during the treatment of naphthalene-contaminatedsoil slurry. ▲, autoclaved soil slurry inoculated with *P. putida* G7, ●, soil slurry inoculated with *P. putida* G7, ■, non-inoculated soil slurry.

The result indicates that all the naphthalene was bioconverted, although, the chemical analysis was performed from a separate set of experiment. Most of the naphthalene was completely mineralized, however, since carbon is also bound to the mass of proliferating cells in the biodegradation process, 100% mineralization is normally not observed (Alexander 1999). Bioconversion into organic intermediates may have occurred, too. No mineralization was observed in the abiotic controls; the naphthalene concentration was still 30 mg l^{-1} at the end of the study, implying that no abiotic degradation occurred during the course of the study.

In addition to the mineralization data, the analysis of 16S rDNA-gene diversity by the PCR-DGGE method confirmed that inoculation had been successful. *P. putida* G7 dominated the microbial community in the inoculated soil slurry (Figure 2b). According to the hybridization analysis, growth of *P. putida* G7 was rapid (Figure 3). In the inoculated soil slurry the numbers of the *nah*A gene increased from below the detection limit to 2.7×10^9 copies μg^{-1} DNA in 6 h, reaching a maximum of 3.5×10^9 copies μg^{-1} DNA close to the end of the exponential mineralization phase after 9 h. After 24 h, the copy numbers of the *nah*A gene started to decrease. The DGGE analysis also showed that the dominance of *P. putida* G7 decreased towards the end of the study and other dominant microbial species appeared in the community.

Even though the number of *nah*A genes was below the detection limit in the native soil slurry at the beginning of the study, there was an increase in the gene numbers during the course of study (Figure 3). The copy number started to increase before any mineralization was observed. A similar significant selection towards a limited number of dominated species occurred before naphthalene mineralization started. The requirement of significant changes in the microbial communities before biodegradation occurred seems to be the reason for the 28 h-long lag period. During the most intensive mineralization stage, additional species appeared in the dominant microbial community (Figure 2a). According to the BLAST search, the main dominant bacteria were related to *Pseudomonas* species (Table 2). The number of *nah*A-like genes increased gradually as the biodegradation process continued in the native soil slurry, but the rate was

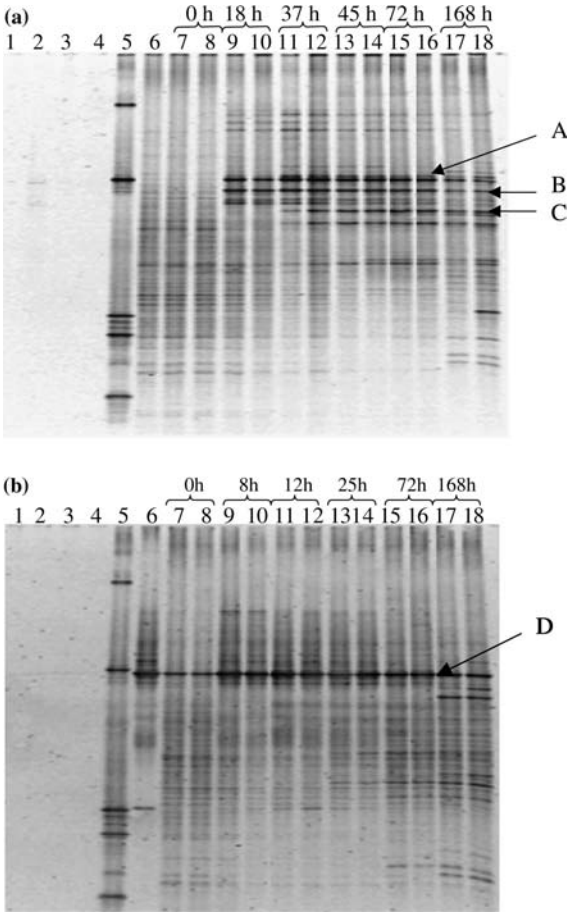

Figure 2. (a) Microbial community in the non-inoculated soil slurry: lanes (1–4) negative controls, (5) DNA marker, (6) Direct soil extract, (7–18) soil slurry 0–168 h. Bands: (A–C) *Pseudomonas* sp. (see Table 2), (b) Microbial community in the soil slurry inoculated with *P. putida* G7: lanes (1–4) negative controls, (5) DNA marker, (6) *P. putida* G7, (7–18) inoculated soil slurry 0–168 h. Band (D) *P. putida* G7.

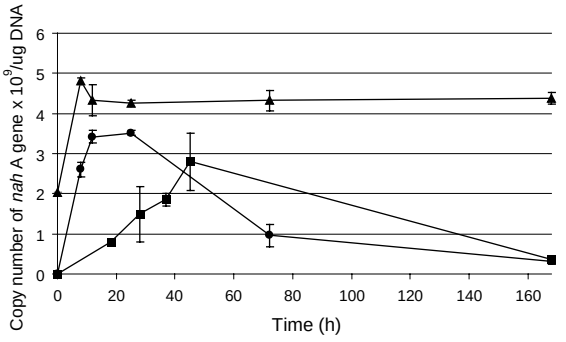

Figure 3. Copy number of *nah*A genes during the soil slurry treatment. ▲, autoclaved soil slurry inoculated with *P. putida* G7, ●, soil slurry inoculated with *P. putida* G7, ■, non-inoculated soil slurry.

Table 2. Sequence similarity between the dominant 16S rDNA-bands and the GeneBank sequences

Band	Bacterial group	Sequence similarity
	Example species (GenBank id number)	
A	*Pseudomonas* sp.	100%
	P. mendelii (gi3065756)	
	P. lini (gi14517354)	
	P. fluorescens (gi18076620)	
B	*Pseudomonas* sp.	98%
	P. syringae (gi28855576)	
	P. putida (gi21635656)	
	P. viridiflava (gi 27884025)	
C	*Pseudomonas* sp.	97%
	P. aeruginosa (gi9951690)	
	P. putida (gi26557018)	
	P. struzeri (gi22474451)	

lower than that in the inoculated soil (Figure 3). The maximum copy number of 2.9×10^9 copies μg^{-1} DNA was observed in the 48 h sample, when the mineralization rate had started to slow down. As *nah*A-like genes are commonly detected in *Pseudomonas* species (Ferrero et al. 2002), the increase in *nah*A genes in the non-inoculated soil slurry is in good agreement with the simultaneous enrichment of *Pseudomonas* species observed in the microbial community analysis. Enrichment of *Pseudomonas*-type organisms in the course of the biodegradation of naphthalene indicates their importance as common degraders of pollutants in various environments, as observed also by Mayer et al. (1999), Plaza et al. (2003) and Margesin et al. (2003). In our study, however, the soil suspension in mineral medium may have promoted the selection towards *Pseudomonas* species.

As the indigenous microbial community in the autoclaved slurry was destroyed, the initial proportion of *nah*A genes in the total DNA extracted from the slurry was high, 2×10^9 copies μg^{-1} DNA. After 6 h 4.3×10^9 copies μg^{-1} DNA were detected (Figure 3). The copy number remained at the same level up until the end of the study, because microbial succession of indigenous organisms was inhibited by autoclavation of the soil slurry. In the other two non-autoclaved treatments, on the other hand, new dominant species appeared in the bacterial community after mineralization had slowed down (Figure 2). As a result

of this succession the number of *nah*A genes started to decrease, and at the end of the 7-day study 0.2×10^9 copies of *nah*A per μg DNA were detected in both the inoculated and non-inoculated soil slurries.

Our results showed that the indigenous microbial community in the PAH-contaminated soil has the potential to biodegrade naphthalene in suitable environmental conditions. However, inoculation of bacteria previously adapted to naphthalene-degradation can significantly enhance the biodegradation of naphthalene during the bioslurry treatment by shortening the lag time that indigenous organisms require to adapt for biodegradation. Similarly, Whyte et al. (1999) reported that a rich consortium previously isolated from contaminated soil significantly shortened the acclimation period for biodegradation of hexadecane. According to Ramirez et al. (2001), successful bioremediation of pyrene-contaminated soils can be achieved by inoculation of soil slurries with pure cultures. However, they speculated that a microbial consortium isolated from contaminated soils may lead to even more complete remediation owing to its ability to utilize degradation byproducts as a carbon source. Bioremediation process seems to require a relatively specific microbial population that differs, according to Stephen et al. (1999), significantly from the original bacterial composition. In our study, several bacteria were enriched during the biodegradation phase in the non-inoculated soil slurry, indicating that biodegradation was accomplished by several species. Even though the soil contained bacteria carrying *nah*A-like genes, these indigenous bacteria were not able to compete with *P. putida* G7 pregrown on naphthalene-containing mineral medium.

In this study, we demonstrated that the efficiency of the biodegradation process can be determined by monitoring the number of catabolic genes. Layton et al. (1994) found significant amounts of *bph* genes after the degradation of PCB had reached a relatively steady level in contaminated soil. The study by Ringelberg et al. (2001), in which increased numbers of genes coding for aromatic hydrocarbon degradation were observed during the course of degradation, is in good agreement with our findings. Guo et al. (1997) reported similar changes in the numbers of *nah*A gene during the incubation of gasoline-contaminated soil. However, they did not analyze the changes in hydrocarbon concentrations in the soil and therefore could not compare them with the biodegradation rate. In addition to these studies, in which enrichment of catabolic genes during the course of the biodegradation was demonstrated, we also found that, after the mineralization of the compound has ceased, the numbers of catabolic genes started to decrease as a result of the gradual decrease of biodegradative organisms in the microbial community due to microbial succession.

Conclusions

According to this study, the slurry phase biodegradation process can be shortened by inoculation with contaminant-degrading bacteria previously adapted to reactor conditions. Our results showed that microbial community analysis by the DGGE method seems to be well suited for monitoring the success of inoculation. The finding that the number of *nah*A genes describing the number of naphthalene-degrading bacteria was in good agreement with the mineralization of naphthalene, demonstrates that the efficiency of the bioremediation process can be determined using catabolic gene-based methods. In addition to following slurry phase remediation, these methods may also be useful for monitoring field-scale remediation processes, as studies by Layton et al. (1994) and Stephen et al. (1999) also indicate. However, it must be kept in mind that there are several different types of enzyme responsible for the degradation of certain compounds (van Beilen et al. 2003), and by merely analyzing a single genotype, does not necessarily sufficiently depict the efficiency of the process. Additionally among the similar genes there tends to be significant sequence diversity between strains as demonstrated for the alkane hydroxylase genes (Smits et al. 1999), and for the *nah*Ac encoding naphtahlene dioxygenase (Ferrero et al. 2002). Therefore, the simultaneous monitoring of other relevant genes, as well as several variations of the same gene, is recommended in hybridization analysis.

Acknowledgements

We would like to thank Anu Kapanen M.Sc. for valuable discussions. Jan Österbacka M.Sc. from Ekokem Palvelu Oy (Finland) is thanked for

providing soil samples, Mrs. Anna-Liisa Ruskeepää for the GC analyses and Dr. Marja Ojala from VTT Processes for determining the PAHs in soil. The research was financed by the Neste Foundation (Finland), Ekokem Foundation (Finland) and VTT's (Technician Research Centre of Finland) Clean world Research Programme. Prof. Gary Sayler and his research group from the University of Tennessee, Knoxville are thanked for the introduction to functional analysis of microbial communities.

References

Alexander M (1999) Biodegradation and Bioremediation. 2nd edn. Academic Press, California, USA, 453 p

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389–3402

Cavalca L, Colombo M, Larcher S, Gigliotti C, Collina E & Andreoni V (2002) Survival and naphthalene-degrading activity of *Rhodococcus* sp. strain 1BN in soil microcosms. J. Appl. Microbiol. 92: 1058–1065

DiGrazia PM, King JM, Blackburn JW, Applegate BA, Bienkowski PR, Hilton BL, & Sayler GS (1991) Dynamic response of naphthalene biodegradation in a continuous flow soil slurry reactor. Biodegradation 2: 81–91

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

Foster J (1995) Determination of soil pH. In: Alef K & Nannipieri P (Eds) Methods in Applied Soil Microbiology and Biochemistry, (p 55). Academic Press, London, UK

Guo C, Sun JB, Harsh JB & Ogram A (1997) Hybridization analysis of microbial community DNA from fuel oil-contaminated and non-contaminated soil. Microb. Ecol. 34: 178–187

Hallier-Soulier S, DucrocQ V, Mazure N & Truffaut N (1996) Detection and quantitation of degradative genes in soils contaminated by toluene. FEMS Microbiol. Ecol. 20: 121–133

Head IM, Saunders JR & Pickup RW (1998) . Microb. Ecol. 35: 1–21

Hwang S & Cutright TJ (2002) Biodegradability of aged pyrene and phenanthrene in a natural soil. Chemosphere 47: 891–899

Layton AC, Lajoie CA, Easter JP, Jernigan R, Sanseverino J & Sayler GS (1994) Molecular diagnostics and chemical analysis for assessing biodegradation of polychlorinated biphenyls in contaminated soils. J. Ind. Microbiol. 13: 392–401

Margesin R, Labbe D, Schinner F, Greer CW & Whyte LG (2003) Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine Alpine soils. Appl. Environ. Microbiol. 69: 3085–3092

Mayer S, Moser R, Neef A, Stahl U & Kämpfer P (1999) Differential detection of key enzymes of polyaromatic-hydrocarbon-degrading bacteria using PCT and gene probes. Microbiology 145: 1731–1741

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

Nano G, Borroni A & Rota R (2003) Combined slurry and solid-phase bioremediation of diesel contaminated soils. J. Hazard. Mat. B 100: 79–94

Plaza G, Ulfig K & Brigmon RL (2003) Relationship between soil microbial diversity and bioremediation process at an oil refinery. Acta Microbiol. Pol. 52: 173–182

Plaza G, Ulfig K, Hazen TC & Brigmon RL (2001) Use of molecular techniques in bioremediation. Acta Microbiol. Pol. 50: 205–218

Ramirez N, Cutright T & Ju LK (2001) Pyrene biodegradation in aqueous solutions and soil slurries by *Mycobacterium* PYR-1 and enriched consortium. Chemosphere 44: 1079–1086

Ringelberg DB, Talley JW, Perkin EJM, Tucker SG, Luthy RG, Bouwer EJ & Fredrickson HL (2001) Succession and phenotypic, genotypic and metabolic community chracteristics during *in vitro* bioslurry treatment of polycyclic aromatic hydrocarbon-contaminated sediments. Appl. Environ. Microbiol. 67: 1542–1550

Sambrook J & Russel DW (2001) Molecular Cloning-A Laboratory Manual. 3rd edn. Cold Spring Harbor Press, Cold Spring Harbor, New York

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

Simon MJ, Osslund TD, Saunders R, Ensley BD, Suggs S, Harcourt A, Suen W, Cruden DL, Gibson DT & Zylstra GJ (1993) Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB9816-4. Gene 127: 31–37

Smits TM, Röthlisberg M, Witholt B & van Beilen J (1999) Molecular screening for alkane hydoxylase genes in Gram-negative and Gram-positive strains. Environ. Microbiol. 1: 307–317

Stephen JR, Chang Y-J, Gan Y-D, Peacock A, Peacock SM, Pfiffner SM, Barlecona MJ, White DC & Macnaughton SJ (1999) Microbial characterization of a JP-4 fuel contaminated site using a combined lipid biomarker/ PCR-DGGE based approach. Environ. Microbiol. 1(3): 231–241

van Beilen JB, Li Z, Duetz WA, Smits THM & Witholt B (2003) Diversity of alkane hydroxylase systems in the environment. Oil Gas Sci. Technol. 58: 427–440

Watanabe K & Baker PW (2000) Environmentally relevant microorganisms. J. Biosci. Bioeng. 89: 1–11

Wenk M, Baumgartner T, Dobovsek J, Fuchs T, Kucsera J, Zopfi J & Stucki G (1998) Rapid atrazine mineralization in soil slurry and moist soil by inoculation of an atrazine-degrading *Pseudomonas* sp. strain. Appl. Microbiol. Biotechnol. 49: 624–630

Whyte LG, Bourbonniere L, Bellerose C & Greer CW (1999) Bioremediation assessment of hydrocarbon-contaminated soils from the high arctic. Bioremed. J. 3: 69–79