Application of microbial community profiling and functional gene detection for assessment of natural attenuation of petroleum hydrocarbons in boreal subsurface

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Received 22 Dec. 2010, final version received 23 Aug. 2011, accepted 11 Aug. 2011


Microbial community structure and functional gene diversity were assessed in subsurface soil and groundwater samples collected from a previously remediated site with residual oil contamination. Primers for functional gene detection and enumeration were designed and tested in order to better quantify pollutant degradation potential in the subsurface soil and groundwater. Results indicate that the study area contains variety of bacteria with capacity to degrade monoaromatic solvents (BTEX), alkanes, low molecular weight PAHs and phenols. Functional genes related to BTEX, phenol and alkane degradation were widely distributed and were found to be especially abundant in zones with higher residual contamination. Results suggest that the indigenous subsurface microbial community at the study site has versatile catabolic potential to degrade different oil compounds. This characteristic is an important prerequisite for the application of natural contaminant attenuation and the successful monitoring of this approach for site remediation.

Introduction

Contamination of environment through the accidental or incidental release of crude and refined petroleum products is a well known worldwide problem. Until the 1990s, it was common practice in many countries to dispose of oily wastes in landfills, which led to numerous soil and groundwater contamination problems (Salminen et al. 2004, Schneider et al. 2006). While many of these former dump sites have been closed and covered, and some have gone through active pump-and-treat remediation, serious oil contamination hazards remain (Röling et al. 2001, Kuchovsky and Sracek 2007). Crude oil and oil products are complex mixtures of various hydrocarbons and associated compounds (Tissot and Welte 1984). The ability of microbes to degrade the main constituents of oil products such as alkanes, BTEX (benzene, toluene, ethylbenzene, xylene) and polycyclic aromatic hydrocarbons (PAHs) has been demonstrated (van Beilen et al. 2003, Meckenstock et al. 2004, Hendrickx et al. 2006a, 2006b), even in cold environments such
as alpine soils (Margesin et al. 2003) or boreal subsurface soils (Salminen et al. 2004).

Using indigenous microorganisms to degrade pollutants as part of the site-recovery process is called monitored natural attenuation (MNA). Natural attenuation is defined as the reduction in toxicity, mass and/or mobility of a contaminant without human intervention owing to both physical and biological processes. In order to verify whether natural attenuation is ongoing and sustainable, the associated processes are monitored over time (Röling and van Verseveld 2002). MNA is regarded as especially effective when combined with active remediation methods and/or when active methods are no longer feasible because of economic or logistic limitations (USEPA 2007) and it has been shown to be functioning at cold-climate sites (Armstrong et al. 2002). Still relatively small number of studies has evaluated the efficacy of MNA as the last or only step in remediation of oil pollution in cold environments and it has mostly been done relying on hydrogeochemical measurements (Van Stempvoort and Biggar 2008). Yet a few studies of cold-climate remediation projects have also reported targeting indigenous microbial communities using molecular methods such as identification of microbial community members (Erikkson et al. 2006), community structure profiling (Labbé et al. 2007) or quantitative PCR for catabolic gene enumerations (Powell et al. 2006).

At the same time, it is accepted that bioremediation cannot be efficiently monitored through a single parameter (Diplock et al. 2009). More in-depth knowledge about the microbial community responsible for bioremediation can be gained by combining community structure analyses with functional gene detection and enumeration using a wide assortment of primer sets targeting various genes linked to contaminant degradation.

The goal of the present study was to simultaneously assess microbial community structure and functional gene diversity at a site with residual oil contamination undergoing MNA treatment. At preliminary stage of the study subsurface soil samples were targeted in order to characterize and zone the study site; in the second phase of the study emphasis was put on investigation of microbial communities of groundwater samples which are commonly used material for monitoring on-going natural attenuation processes.

**Material and methods**

**Field site history**

Subsurface soil and groundwater samples were collected at the Lägja landfill, southern Estonia where municipal and industrial wastes has been deposited since the early 1970s (Fig. 1). Since the mean air and soil temperatures for the period 2001–2010 were 6.3 °C and 5 °C, respectively (data source Estonian Meteorological and Hydrological Institute), according to Stempvoort and Biggar (2008) the landfills location could be classified as a cold-climate site. When the landfill closed in 2004 it covered 1.4 ha and contained about 50 000 tonnes of municipal waste. A shallow, 1-ha pond with no outlet was located in the lowermost section of the landfill. Fuel tank sediments, bilge water, various kinds of oily waste and oil-contaminated water were dumped into the pond from 1974 to 1993. The pond also received landfill leachate and surface runoff from the surrounding drainage area.
In 2002–2004, the integrated remediation plan of the Laguja landfill was conducted and included (i) removal and treatment of oily leachate from the pond, (ii) removal and treatment of oily sediments, (iii) filling two smaller empty (no water) pond segments with inert demolition waste, (iv) profiling and capping of the landfill, and (v) creation of a constructed wetland for further treatment of the leachate. Despite these actions, residual oil contamination was still present at the site at the time of the study (2004–2008). Initial chemical analysis of subsurface soil samples revealed average residual total petroleum hydrocarbons (TPH) contamination of 80 mg kg$^{-1}$ at the field site, whereas some hotspots receiving landfill leachate had TPH concentrations of up to 960 mg kg$^{-1}$. Subsequently collected groundwater samples were subjected to more thorough chemical analyses (Table 1) (Eurofins Analytico B.V., Netherlands). Monitoring wells P1 and P2 had been installed at the field site prior to this study and had been considered non-polluted reference sites. We adopted the same approach as these wells were showing only minute traces of oil products (Table 1).

**Subsurface soil and groundwater sampling**

Subsurface soil samples for preliminary site characterisation and zoning were obtained from fresh sediment cores taken during the installation of groundwater monitoring wells around the pond (Fig. 1). The wells installed between 1 and 4 November 2006 were 3-m deep and six samples were taken from different depths (0.3–0.5 m, 0.8–1.0 m, 1.3–1.5 m, 1.8–2.0 m, 2.3–2.5 m, 2.8–3.0 m). The wells installed on 5 and 6 September 2007 were 2-m deep and four samples were taken (0.3–0.5 m, 0.8–1.0 m, 1.3–1.5 m, 1.8–2.0 m). Samples were stored in sterile plastic boxes at 4 °C; subsamples for molecular biological analyses were immediately frozen and stored at −80 °C until DNA extraction.

Before groundwater sampling the monitoring wells were pumped until empty. Up to 2000 ml of fresh groundwater seepage were collected from the maximum depths of monitoring wells 1, 4, 6 and P2 in September 2008 and from borehole P1 and the pond (grab sample) in October 2008 (Fig. 1). Groundwater samples from boreholes 3 and 5 could not be collected due to dissatisfactory technical state of these monitoring wells. Samples were stored in sterile 2-l glass bottles at 4 °C during transport and filtered in the laboratory within three hours of arrival using 0.2 µm filters, which were stored at −80 °C until DNA extraction.

**Enrichment cultures and isolation**

For enrichment cultures, 50 ml of Bushnell-Haas (BH) minimal medium was used. Crude oil (0.5 or 2.5 ml), diesel fuel (0.5 or 2.5 ml) or hexadecane (0.5 or 2 ml) served as the sole carbon and energy source for growth. One gram of sediment from borehole 3 (depth 0.3–0.5 m), borehole 5 (depth 0.3–0.5 m) or borehole 5 (depth...
1.3–1.5 m) was added to the enrichment culture mixture and incubated aerobically at 15 °C with shaking at 180 rpm for 15 days.

To obtain xylene-degrading isolates, the post-incubation cultures were diluted up to five times in BH medium and plated on minimal medium agar plates. A glass capillary tube containing para-xylene was attached to the petri dish lid. After six days of incubation in xylene vapours at room temperature, colonies were plated onto replicate minimal medium agar plates, each with a xylene-containing capillary tube attached to the petri dish lid.

To obtain alkane-degrading isolates, the post-incubation cultures were diluted up to seven times in BH medium and inoculated to microtiter plates in 200 µl BH medium with 5 µl hexadecane in five repetitions. After eight days of incubation at room temperature, 20 µl of INT [2-(4-jodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride] (Acros Organics, Belgium), at a concentration of 3 g l–1, were added to each microtiter plate cell. Incubation continued for additional 24 h, after which optical density of samples was measured at 480 nm. Cells which showed formasan production but had low optical density were plated on Luria-Bertani (LB) agar plates.

DNA extraction

The total community DNA was extracted from 0.25 g of each subsurface soil sample obtained from field site using a PowerSoil DNA Extraction Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) and from groundwater filters using an UltraClean MegaPrep Soil DNA Extraction Kit (Mo Bio Laboratories, Inc.). The DNA extractions from borehole 1 (depths of 0.3–0.5 m, 0.8–1.0 m, 2.3–2.5 m and 2.8–3.0 m) were completed on 10 g of soil using the UltraClean MegaPrep Soil DNA Extraction Kit (Mo Bio Laboratories, Inc.).

Total genomic DNA from enrichment cultures and bacterial strains grown on minimal medium agar plates exposed to xylene vapours or on LB agar plates was extracted using an UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc.). All DNA extractions were completed according to the manufacturer’s instructions.

PCR amplifications of catabolic genes

The DNA extracts were screened by polymerase chain reaction (PCR) using the following primer sets for detection of catabolic genes that encode enzymes involved in a variety of known bacterial hydrocarbon degradation pathways: TMOA-F/TMOA-R (Hendrickx et al. 2006a), TBMD-F/TBMD-R, Xyla-F/Xyla-R, TODC1-F/TODC1-R, Xyle1-F/Xyle1-R, Xyle2-F/Xyle2-R, CDO-F/CDO-R (Hendrickx et al. 2006b), TOL-F/TOL-R (Baldwin et al. 2003), universal alkM-F/alkM-R (Margesin et al. 2003), BP-F/BP-R (Sipilä et al. 2006) and Phe00/Phe212 (Watanabe et al. 1998, Heinaru et al. 2005). PCR amplifications were conducted using a 25 µl reaction mixture containing 1 × PCR buffer with (NH₄)₂SO₄ (75 mM Tris-HCl, pH 8.8; 20 mM (NH₄)₂SO₄; 0.01% Tween 20), 2.5 mM MgCl₂, 0.006 mg ml–1 bovine serum albumin (BSA), 0.2 mM of each deoxynucleotide triphosphate, 0.0008 mM (each) of forward and reverse primers, 0.5 U of Taq DNA polymerase (MBI Fermentas, Lithuania) and 15 ng of soil DNA or 40 ng of groundwater DNA template. All PCR amplifications were carried out as described previously (Hendrickx et al. 2006b) and were performed on Eppendorf Mastercycler or Thermal cycler PCR machines. The PCR fragments were analyzed by agarose gel electrophoresis (1% or 2% agarose depending on the length of amplified product) and visualized by ethidium bromide staining. The limit for visually detecting PCR amplification products for the primer sets TODC1-F/TODC1-R and BP-F/BP-R was 10⁵–10⁶ gene copies/g of soil (Hendrickx et al. 2006b, Sipilä et al. 2006). The detection limit for all other primer sets was 10³–10⁴ gene copies/g of soil (Watanabe et al. 1998, Margesin et al. 2003, Hendrickx et al. 2006b). Secondary PCR was performed by using 0.5 µl of the primary product from the first PCR as a template. All experiments included control reaction mixtures without added DNA.

16S rRNA gene sequencing

16S rRNA genes from xylene- and alkane-degrading isolates were PCR-amplified from
genomic DNA with primers PCRI and PCRRII (Weisburg et al. 1991). All other components and concentrations in the master mix were the same as described above and amplification was performed as described previously (Weisburg et al. 1991). Amplified PCR products were purified using 2.5 U ExoI and 1 U SAP enzymes (USB Corporation, Cleveland, OH, USA) for 15 min at 37 °C followed by enzyme denaturation for 15 min at 80 °C. Purified products were sequenced with primers SEQ1 and SEQ2 (Vedler et al. 2000). Partial 16S rRNA gene sequences were obtained using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 377 DNA Sequencer (Perkin-Elmer, Waltham, MA, USA). All partial 16S sequences were compared with those in available databases using the BLAST program (NCBI, URL http://blast.ncbi.nlm.nih.gov).

Denaturing gradient gel electrophoresis (DGGE) and data analysis

Prior to DGGE, DNA samples from subsurface soil, groundwater and enrichment culture communities and from bacterial isolates were subjected to PCR amplification. PCR amplifications were conducted with primers P338F-GC/P518R (Øvreås et al. 1997) and 0.5 µl of template from the primary PCR product. All other components and concentrations in the master mix were the same as described above. Amplification was performed as described previously (Øvreås et al. 1997). To separate the amplified gene fragments, either the DCode Universal Detection System (Bio-Rad, Hercules, CA, USA) or INGENYphorU-2 × 2 (Ingeny International, Netherlands) electrophoresis system was used, as recommended by the manufacturer. Approximately 100 ng of PCR products were applied for the DGGE analyses and electrophoresis was performed as described previously (Øvreås et al. 1997). To separate the amplified gene fragments, either the DCode Universal Detection System (Bio-Rad, Hercules, CA, USA) or INGENYphorU-2 × 2 (Ingeny International, Netherlands) electrophoresis system was used, as recommended by the manufacturer. Approximately 100 ng of PCR products were applied for the DGGE analyses and electrophoresis was performed as described previously (Muyzer et al. 1993) with 10% (vol/vol) polyacrylamide gel (acrylamide:bisacrylamide = 37.5:1 in 1× TAE buffer). A DNA denaturing gradient was formed with deionized formamide and urea (100% denaturant gradient is 7 M urea and 40% (vol/vol) deionized formamide); a linear denaturing gradient of 35%–70% was used. Electrophoresis was accomplished using 1× TAE buffer at constant voltage (100 V) and temperature (60 °C) for 12 h. Bands were visualized by staining them in MilliQ water (Millipore, Billerica, MA, USA) with 1× SYBR Gold (Molecular Probes, OR, USA). DGGE gels were digitized and the banding pattern evaluated by cluster analysis based on Pearson’s correlation coefficient using GelComparII ver. 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). A canonical analysis of principal coordinates (CAP) was performed on the correlation matrix using a permutation test of significance, and the leave-one-out approach to estimate goodness of fit of the groups formed during clustering procedure (Anderson and Willis 2003). In order to compare the dispersions among groups the distance-based test for homogeneity of multivariate dispersions was carried out (Anderson 2006).

Quantitative PCR

Primer sets 785FL/919R, Phe00L/Phe212 and alkMF2/alkMRL (Table 2) were used for 16S rRNA, LmPH and alkM gene detection and enumeration on SYBR green qPCR. Primer set λ7403FL/λ7512R (Table 2) was used for PCR inhibition measurement. Primers were designed or modified manually following LUX primer design rules (Nazarenko et al. 2002), primer properties were calculated with OligoAnalyzer 3.0 software (Integrated DNA Technologies, IA, USA) and the specificity of primer pairs was checked by sequence alignment using BLAST and NCBI entries.

For standard curve creation DNA of reference strains Pseudomonas mendocina PC1 for 16S rRNA and LmPH genes and Acinetobacter sp. T3 for alkM gene was PCR amplified. The PCR reaction mixture was prepared as described above with the exclusion of BSA from the mixture. The PCR reactions were performed with following reaction conditions: preheating at 95 °C for 5 min; 30 thermal cycles of 30 s at 94 °C, 30 s at annealing temperature of a primer pair used (Table 2) and 45 s at 72 °C. The PCR products gained were cloned using InstA/Aclone PCR cloning kit (Fermentas) according
to manufacturer’s instructions and plasmid DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen, CA, USA) and nucleotide sequenced using BigDye™ chemistry with M13 primers (Fermentas). The number of copies of standard plasmids were calculated according to plasmid (2886 bp) plus insert lengths (Table 2), assuming a molecular mass of 660 Da for a base pair. Standard DNA stock solutions of $10^9$ plasmid copies µl$^{-1}$ were prepared and serial dilutions ranging from 25 to $10^8$ target gene copies were used for standard curve creation on qPCR. The detection limit for all assays was 25 target gene copies per 1 µl of template.

The qPCR assays were performed on the real-time PCR system Rotor-Gene® Q (Qiagen) and data was analysed using Rotor-Gene Series software, version 2.0.2. Optimized reaction mixture contained 5 µl Maxima SYBR Green Master Mix (Fermentas); 0.0002 mM of forward and reverse primer, 1 µl template DNA and 3.6 µl sterile distilled water adding up to the total volume of 10 µl. The optimized reaction conditions were: 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 30 s at 63 °C and 30 s at 72 °C. Immediately after the real-time PCR assay, melting curve analyses was performed ramping temperatures from 65 °C to 90 °C using 3 second and 0.35 °C interval with continuous fluorescence recording. The initial target gene copy number in environmental samples was deduced from the standard curves. The presence of PCR inhibitors was evaluated by mixing 1 µl of environmental DNA with 1 µl of $10^4$ copies of the lambda-standard plasmid. When recovery of lambda DNA differed from 100%, the quantification data was corrected using the corresponding efficiency factor.

## Results

### Bacterial strain isolation and identification from the enrichment experiment

Following eight days of growth in the enrichment cultures, serial dilutions were inoculated to microtiter plates with hexadecane as the sole carbon source. Enrichment culture serial dilu-
tions were also plated onto solid media and grown with xylene vapours as the sole carbon source. Fourteen different bacterial isolates, presumed to be able to significantly utilize petroleum hydrocarbons as a carbon source, were isolated. In order to identify the genera of the obtained isolates (99%–100% identity), the 16S rRNA gene was amplified and the partial (approximately 1000 bp) sequences were obtained and compared with those available in GenBank using the BLAST program.

Three obtained isolates were matched to the genus *Sphingopyxis*, three were matched to *Pseudomonas* and two were matched to *Acinetobacter* (Table 3: upper section). *Stenotrophomonas*, *Gordonia*, *Acidovorax* and *Arthrobacter* had one matching isolate each. Two isolates showed less than 99% identity to GenBank sequences (98% and 94%, respectively); the closest matches belonging to the genus *Dyadobacter*.

### Microbial community profiling with DGGE

Subsurface soil, groundwater and enrichment culture microbial communities were investigated using DGGE profiling of 236 bp fragments encompassing the variable region V3 of 16S rRNA genes amplified from genomic DNA and subsequent dendrogram generation based on DGGE banding patterns (Fig. 2).

The subsurface soil samples clustered into three major groups, based primarily on sam-

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<th>Samples</th>
<th>BTEX degradation initiation</th>
<th>BTEX aromatic ring cleavage</th>
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<td><strong>tbmD</strong></td>
<td><strong>tmoA</strong></td>
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<td><em>Pseudomonas</em></td>
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4S | – | + | + | – | – | + | – | – | – | – | + |
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P1W | + | – | – | – | – | – | – | – | – | – | – |
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1 Maximum GenBank identity 94%, 2 Maximum GenBank identity 98%.
pling location; however, many samples in these clusters showed low similarity to each other suggesting heterogeneous conditions and microbial communities at the field site (Fig. 2a). A permutation test \(( p < 0.0001, 9999 \text{ permutations})\) and a leave-one-out cross-validation test (misclassification error 0\%) revealed significance of dividing the soil samples into three clusters. The first cluster on the dendrogram consists of four samples showing high diversity with no dominant bands from borehole 1 which had several distinctive oily layers along the depth profile. The second cluster links samples from boreholes 3 and 4 as well as two layers from borehole 1. This group showed in some part higher similarity and emergence of small number of dominant bands. The third cluster links samples from boreholes 5 and 6 which were exposed to landfill leachate. However, these community profiles showed no dominant bands and had low similarity to each other once again hinting heterogeneous conditions at field site. A distance-based test for homogeneity of multivariate dispersions revealed significant difference \(( p < 0.05, 9999 \text{ permutations})\) in dispersion between clusters II and III indicating the heterogeneity of study area. Comparison of groundwater and selected subsurface soil DGGE profiles indicated a two-cluster separation where all groundwater microbial community profiles clustered to one branch of a dendrogram (Fig. 2b). The groundwater profiles differed significantly from the subsurface soil communities but also showed very high variability within their own cluster.

As expected, the community profiles of the enrichment cultures showed decrease in diversity and the emergence of a small number of dominant bands (Fig. 3). A review of bacterial isolate DGGE profiles shows that the isolate bands are represented in the DGGE profiles from subsurface soil samples which were exposed to contaminated landfill leachate. The isolates do
not, however, dominate the microbial communities. In the enrichment cultures, on the other hand, profile bands corresponding to the isolates, particularly those belonging to *Pseudomonas*, *Acinetobacter* and *Sphingopyxis*, were among the dominant taxa.

**Detection of functional genes**

In order to assess catabolic potential of the indigenous microbial communities, 11 different primer sets were used to detect functional genes involved in hydrocarbon degradation pathways. The total community DNA from all the subsurface soil and groundwater samples and DNA from bacterial isolates were screened using a semi-nested PCR approach. In addition to phenol and alkane hydroxylase genes, several targeted genes originate from BTEX and PAH degradation pathways.

Functional gene detection from subsurface soil and groundwater samples showed that *LmPH* was the gene most frequently detected in all samples without any exceptions; *xylM*, *xylE1*, *tmoA* and *alkM* were detected in soil from all boreholes but generated a signal from only a few groundwater samples (Table 3: lower section). The other widespread catabolic genes were *tbmD* and *cdo*, which were absent only from borehole 4 and borehole 3 soil samples, respectively. Other genes, such as *nahC*-like and *xylE2* genes were encountered on a more random basis. *XylA* and *todC1* genes showed the fewest occurrences, being detected only in soil samples exposed to contaminated landfill leachate (boreholes 5 and 6) and a borehole 4 groundwater sample, respectively. Functional gene detection results obtained from bacterial isolates corresponded exceptionally well to functional gene detection results from environmental samples. *LmpH*-*, *tmoA*-*, *xylM*-*, *xylE1*- and *alkM*-like genes were detected frequently, whereas *tbmD*- and *cdo*-like genes were detected only in one and two isolates, respectively (Table 3: upper section). The *todC1*-*, *xylE2*- and *xylA*-like genes were not detected in isolates.
Total bacteria and functional population enumeration in groundwater using qPCR

16S rRNA and two functional genes, *LmPH* coding large subunit of multicomponent phenol hydroxylases and *alkM* coding alkane hydroxylases, were targeted on screening of groundwater samples using SYBR green qPCR approach. Due to the presence of PCR inhibitor substances in environmental DNA, similarly to previous reports (Cébron *et al.* 2008) data were corrected using the bacteriophage lambda internal standard during qPCR assays. The phage lambda DNA recovery rates for groundwater samples were 72.2%–100%, and for a few tested soil samples (quantification data not shown) recovery rates were 73.6%–100%. Nucleotide sequences obtained from the reference strain and environmental samples showed > 99% similarity to the corresponding 16S rRNA, *LmPH* and *alkM* sequences in GenBank.

The 16S rRNA gene copy numbers ranged from $2.7 \times 10^5$ to $2.5 \times 10^7$ copies ml$^{-1}$ groundwater (Fig. 4a). Up to two orders of magnitude higher 16S rRNA gene copy numbers were detected in groundwater monitoring wells with residual oil contamination compared to uncontaminated wells. Detected *LmPH* gene copy numbers ranged from 12 to 465 genes ml$^{-1}$ of groundwater at different sampling locations (Fig. 4a) and showed the similar trend of abundance towards wells with residual contamination (except borehole 1) as 16S rRNA genes. For easier comparison of the functional populations among boreholes, the *LmPH* gene levels were normalized as a percentage of the entire community according to 16S rRNA copy number (Fig. 4b). *Alkm* genes could not be detected in any analysed samples even though chemical data from the field site showed alkanes of different chain lengths to be predominant pollutants remaining in groundwater.

**Discussion**

Among the processes affecting the course of MNA, biodegradation is the major mechanism that helps to significantly reduce and/or eliminate the residual contamination. Assessment of microbial community characteristics of polluted soil and groundwater provides important information about the suitability of the MNA approach for treatment of residual oil contamina-
tion in the respective media. This kind of information should prove especially useful regarding bioremediation at cold-climate sites where biodegradation processes are considerably slower than in temperate environments.

Although only a small proportion of bacterial taxa can be successfully cultured and those isolated bacteria do not represent the actual catabolic capacity of the polluted site (Torsvik et al. 2002), this exercise still provides valuable information about the indigenous bacterial genera present at the field site. Sequencing the 16S rRNA gene from the bacterial isolates demonstrated that some of the closest GenBank matches to the acquired sequences belong to genera which are well known as hydrocarbon degraders and often dominate the bacterial communities at oil-polluted sites. Example genera include Pseudomonas, Acinetobacter (Van Hamme et al. 2003) and Sphingopyxis (identified as a distinct genus from Sphingomonas with which it was previously grouped) (Takeuchi et al. 2001). Other sequences matched recently discovered taxa such as Gordonia and Acidovorax, which have also been identified as petroleum hydrocarbon degraders, although detailed ecological and taxonomic descriptions are unavailable (Monferran et al. 2005, Quatrini et al. 2008). Because of the petroleum-biodegrading capacity of the taxa identified in this study, as well as the potential variability among the genera, additional investigation (substrate range, degradation kinetics, usage of different electron acceptors) of these isolates is warranted. The isolated petroleum hydrocarbons degrader’s importance in field site cannot be evaluated on basis of isolations and enrichment cultures due to cultivation bias. Therefore, the specific role of isolated degraders as well as their abundance at field site should be addressed in further research. Slightly problematic were two isolate sequences that best matched the genus Dyadobacter but also showed less than 99% similarity to any GenBank sequence. The lower similarity may be because Dyadobacter is not yet well defined (Willumsen et al. 2005) and not many sequences from this genus reside in GenBank; the taxonomic affiliation of those isolates remains to be positively established.

Hydrocarbon contamination stimulates the growth of indigenous catabolic microbes, causing changes in community structure; MNA strategy relies solely on the intrinsic biodegradation capacity of these altered microbial communities (Sipilä et al. 2006). Variable conditions at sampling locations might be reflected in differences in microbial community structure (Andreoni et al. 2004). Based on DGGE banding patterns, the bacterial communities of subsurface soil samples clustered into three major branches on similarity dendrogram indicating differing bacterial community composition at field site. However, many samples in these clusters showed high diversity and therefore low similarity to each other which might suggest rather heterogeneous conditions and microbial communities at the field site. It has to be kept in mind that samples from only five sampling locations were tested and the heterogeneity of results suggests that there could be more than three zones with different conditions and bacterial communities present at the field site. If the distinction of the field site into different zones based on microbial community structure is desirable, more sampling locations should be chosen and tested. The DGGE profiles of groundwater samples did not show the same distinct clustering (as evident in the soil samples) and were more similar to each other than to subsurface soil profiles from the same boreholes. This trend has been noted before (Röling et al. 2001). The heterogeneity of microbial communities present at field site emphasizes the need for detailed catabolic potential characterization to confirm and estimate the potential and functioning of MNA approach.

Selected subsurface soil community and the enrichment-community (DGGE) profiles were compared to the acquired bacterial isolates. Some Gram-negative genera like Pseudomonas or Acinetobacter often show significant increases following oil contamination (Margesin et al. 2003); this proved to be the case in the enrichment cultures. Through DGGE profiling it was also found that most bacterial isolates, notably those belonging to Pseudomonas, Acinetobacter, Sphingopyxis and Acidovorax, are associated with the degradation of crude oil and were present in various subsurface soil samples exposed to contaminated landfill leachate, even though they were not the dominant community taxa. The results indicate that the indigenous
microbial community from the sampling locations has a potential to biodegrade petroleum products. In addition, the enrichment culture experiment indicated that there are several taxa in the community which tolerate higher petroleum concentrations and might promote remediation of even greater amounts of oil contamination than are currently present. The occurrence of condition-adapted indigenous microorganisms that are capable of oil-degradation in the field fulfils one of the prerequisites for successful application of MNA. However, it has to be kept in mind, that other requirements, such as interactions between microorganisms and their geological and hydrological environment, especially the availability of substrates, electron acceptors and nutrients, should be investigated and fulfilled to guarantee the success of MNA approach at field scale (Röling and van Verseveld 2002).

In case of 16S rRNA gene diversity and community structure studies, the function of the retrieved community remains uncertain. Functional marker gene studies can rapidly provide a profile of the genetic diversity of functional genes and microbial community catabolic potential at field sites (Hendrickx et al. 2006b, Sipilä et al. 2006). We used a complex assortment of primer sets targeting various functional genes involved in aerobic alkane, BTEX and PAH degradation pathways which, to date, have been primarily used for addressing BTEX-contaminated subsurface soil (Hendrickx et al. 2006ab) in temperate regions and in Pacific Ocean sediment and water (Wang et al. 2008). The current study demonstrates that these primer sets can also be applied to samples from cold environments. Catabolic genes connected to BTEX, alkane and phenol degradation were detected from all established zones, which indicate that the indigenous bacterial communities have probably been subjected to selective contaminant pressure for several years and, as a result, the proportion of bacterial strains with good catabolic potential has increased. AlkM, LmPH, tbmD-, tmoA-, xylM-, xylE1- and cdo-like genes were detected in abundance at the field site. They were even detected, although to a lesser extent, in boreholes (P1 and P2) considered free of contamination during active remediation that preceded current application of the MNA approach. The fact that xylA did not necessarily co-occur with xylM was affirmed by functional gene detection data from bacterial isolates; this situation has been reported in previous studies (Hendrickx et al. 2006b). NahC-like genes were detected randomly from different field site locations, however, lower detection rates associated with the utilized primers must be considered in the spatial interpretation of gene occurrence (Sipilä et al. 2006). Functional gene detection data from environmental samples correlated well with the data obtained from the isolates in which LmPH-, xylE1-, tmoA-, xylM- and alkM-like genes were most frequently encountered.

Enumeration of 16S rRNA genes gives background information about the total microbial community present at the study site and also enables normalizations of functional gene numbers as compared with the entire community. It is known that contamination can boost the growth of indigenous catabolic microbes (Margesin et al. 2003) which manifests itself also in changes in microbial community abundances. This trend could well be seen in 16S rRNA gene quantification result which showed up to two orders of magnitude higher 16S rRNA gene copy numbers in groundwater monitoring wells with residual oil contamination compared to uncontaminated wells.

The LmPH gene codes the key enzyme for aerobic metabolism of phenol and has been used as a molecular marker over a decade (Watanabe et al. 1998). It has also been established as being important in bioremediation assessment (Heinaru et al. 2005). Quantification of LmPH showed the similar trend of abundance towards wells with residual contamination (except borehole 1) as 16S rRNA genes. This corresponds well to previously reported results (Basile and Erijam 2010). LmPH gene copy numbers in borehole 1 were surprisingly quite low but it has to be taken into consideration that this monitoring well had shown high amounts of total petroleum hydrocarbons which could serve as other preferable carbon sources for indigenous microbes besides phenol. Occurrence of very heterogenous microbial community supports that assumption. On the other hand, LmPH genes were detected in boreholes P1 and P2 which were considered free of contamination during active remediation
that preceded application of the MNA approach. Notably, even though the 16S rRNA and \textit{LmPH} gene copy numbers detected from these boreholes were lower than in most other sampling locations, the normalizations showed proportionally the highest catabolic gene presence in these boreholes. \textit{LmPH} gene normalization results as percentages of the entire community show that the functional populations carrying \textit{LmPH} genes are very evenly distributed over the field site which indicates that the indigenous microbial communities have good and stable biodegradation potential targeting phenol compounds.

To our surprise \textit{alkM} genes could not be quantified in any analysed groundwater samples. The chemical data from the field site had shown alkanes of different chain lengths to be predominant pollutants remaining in groundwater and \textit{alkM} genes were detected from the same groundwater samples using seminested approach of conventional PCR. However, \textit{alkM} genes have very rarely been detected in pristine soils (Margesin \textit{et al.} 2003) and even though in this case alkanes are predominant pollutants remaining at the field site, the pollutant concentrations are rather low. Therefore, it might be the case that the \textit{alkM} genes are present at field site at so low concentrations that they remain under the detection limit of SYBRgreen qPCR assay. The primers used in this study were modified or designed in a way that upon attaching appropriate fluorophore they can also be used on LUX\textsuperscript{TM} qPCR assays if more precise estimation or lower detection limit of target gene copy numbers should prove necessary (Nazarenko \textit{et al.} 2002). This previous assumption should be verified in further studies.

Conclusions

Results indicate that the indigenous microbial community at the field study location possesses a versatile catabolic potential allowing for degradation of various petroleum compounds even in cold-climate conditions, thus fulfilling an important MNA prerequisite. Although oily water is constantly leaching out of the closed landfill, contamination does not appear to be spreading, presumably because of natural attenuation. The complex assortment of primer sets developed previously and in this study can be used as a rapid and informative tool for the functional characterization of the microbial community at oil-contaminated cold-climate field sites. The developed and optimized qPCR assays can be valuable tools for bioremediation monitoring and can be applied to assess the occurrence of target genes also in other types of environments besides groundwater thanks to internal standard method for inhibition evaluation.

Acknowledgements: This research was supported by Estonian Science Foundation grant no. 7827 and by the Estonian Ministry of Education and Research grants nos. SF0180026s08 and SF0180127s08.

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