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Theses of doctoral (Ph.D.) dissertation

COMPARATIVE STUDY OF MICROBIAL COMMUNITIES INVOLVED IN THE AEROBIC AND MICROAEROBIC DEGRADATION OF HYDROCARBONS USING COMPLEX ENRICHMENT CULTURES

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1. Introduction and aims

The growth of population and human needs cause increasing energy demand. Although several alternatives already exist, our energy supply is still largely derived from the burning of fossil fuels such as coal, crude oil, and natural gas. Petroleum derivatives also serve as an important raw material and solvent in the industry and everyday life. Due to accidents and negligence, pollution is very common during extraction, transportation, storage, and usage. It is not surprising, that petroleum derivatives are the most common pollutant in our country and the world as well. Released into the environment, they can cause serious damage in the affected ecosystem since the pollutants can accumulate in animal and plant tissues causing death or mutations. The best solution is prevention; however, in practice, this is often not realized.

If pollution has already occurred, techniques are available to control the problem and, in some cases, to restore the original states. There are three main remediation methods: physical, chemical, and biological (also known as bioremediation procedures). Bioremediation is receiving increasing attention since these are the most environmentally friendly the most cost-effective methods. In bioremediation procedures, bacteria are used to eliminate xenobiotics. Because of the immeasurable diversity of bacteria and the practical limitations of their identification, we know only a fraction of bacterial species. To develop remediation procedures and increase their efficiency, we have to expand our knowledge. While microorganisms "working" under aerobic conditions have already been used successfully in the biodegradation of petroleum pollutants, the role of microorganisms active in microaerobic conditions has received relatively little attention, although they may have a key role in a deep, oxygen-limited environment. Some bacteria have an enzyme system that allows them efficient metabolism even at low dissolved oxygen concentrations. The production of these enzymes is encoded by special functional genes. A better understanding of these

genes can be an effective tool to study the metabolic activity in the environment and the metabolism of some bacteria.

The primary aims of our research are to:

- reveal how different microbial communities evolve in aromatic hydrocarbons or diesel fuel/crude oil-contaminated environments under aerobic and microaerobic conditions;
- determine which type of functional genes involved in the degradation of aromatic and aliphatic hydrocarbons and which components could be decomposed by these genes.

Our main goal was to enrich and isolate strains that are capable to degrade hydrocarbons.

2. Materials and methods

2.1. Conditions of microbial enrichment experiments

To reveal the effect of oxygen limitation on the structure of hydrocarbon-degrading bacterial communities, two enrichment experiments were performed. In both cases, we set up aerobic (7-8 mg/L dissolved oxygen concentration) and microaerobic (≤ 0.5 mg/L dissolved oxygen concentration) enrichments. In one of the experiments, aromatic hydrocarbons (toluene or benzene), in the other experiment, aliphatic hydrocarbons (fuel/crude oil mixture) were use as sole carbon and energy sources for bacteria (Fig. 1).

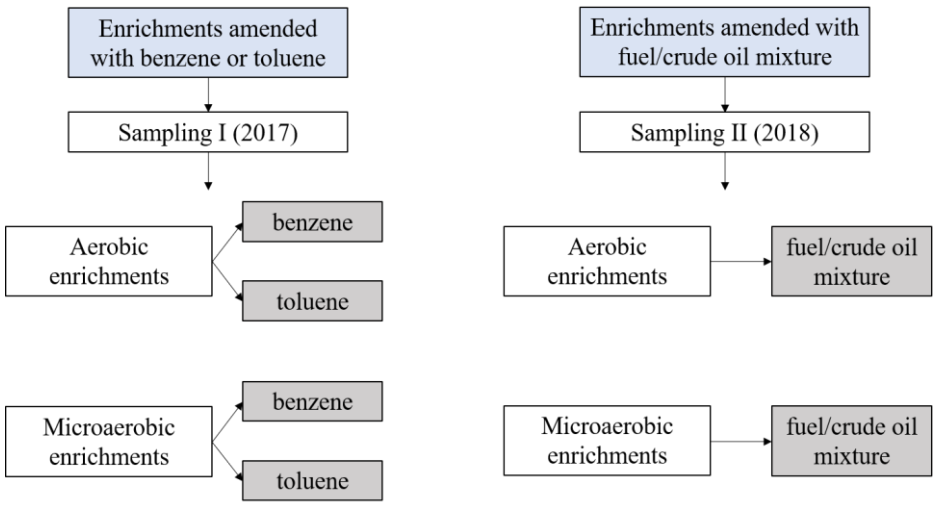

Figure 1: Schematic diagram of enrichment experiments. The text boxes with gray background indicate the type of carbon source used.

2.2. Methods used to reveal microbial community compositions

For the inoculation of the microcosms, a biofilm sample was obtained from a petroleum hydrocarbon–contaminated site located in central Hungary. Freshwater medium amended with vitamins was used for the experiments. The enrichment cultures were transformed weekly for five consecutive weeks, and the

carbon source was monitored by GC-MS during this period. Developed bacterial communities were subjected to microbial studies, see Fig. 2.

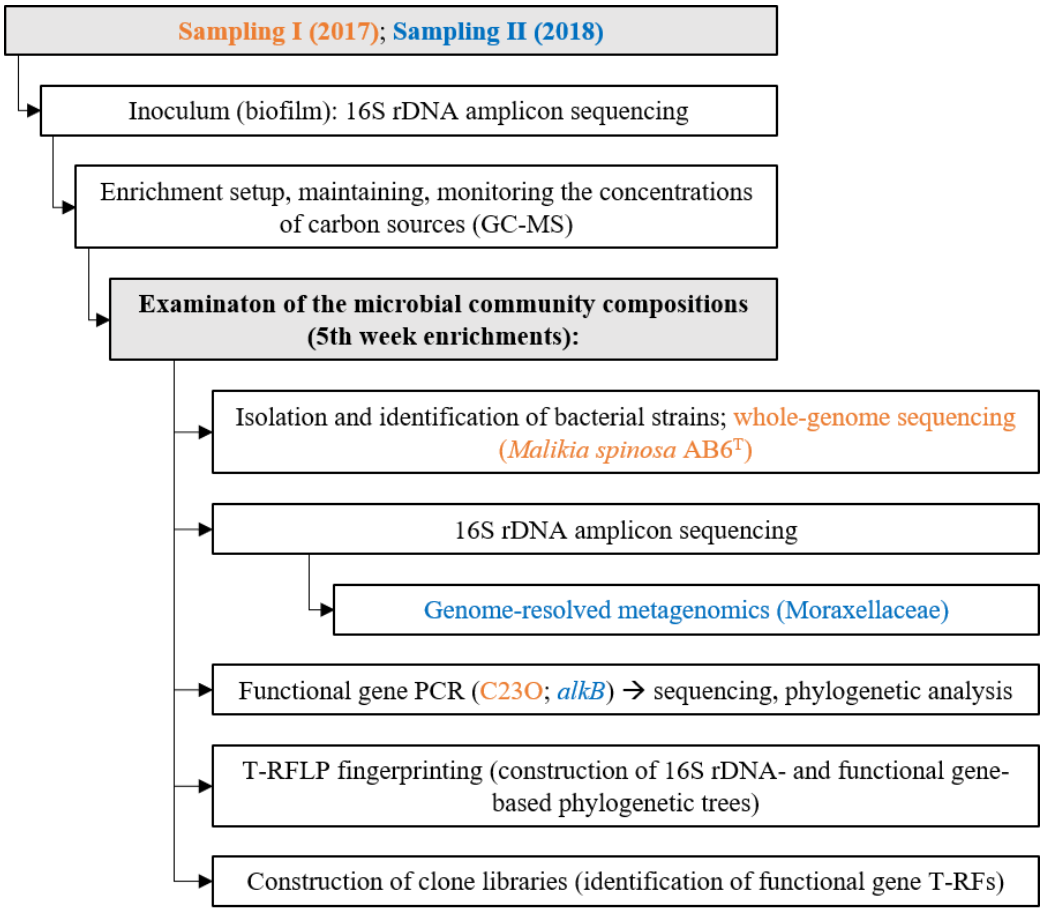

Figure 2: The schematic illustration of the processes related to the enrichment experiments. Black text: methods used in both experiments presented in Fig. 1. Orange text: methods used in case of enrichment cultures amended with benzene or toluene (left-hand side of Fig. 1). Blue text: methods used in case of enrichment cultures amended with fuel/crude oil mixture (right-hand side of Fig. 1).

To assess the bacterial community composition of the initial biofilm sample as well as of the aerobic and microaerobic enrichments of the 5th week, Illumina 16S rDNA amplicon sequencing was carried out. The variable V3 and V4 regions of the 16S rDNA were amplified on Illumina MiSeq platform by Seqomics Kft. For enrichment cultures used to investigate the degradation of

aliphatic hydrocarbons, metagenome sequencing and metagenome-associated genome analysis were performed to identify the unknown *alkB* genotype found in the enrichments. In the aromatic hydrocarbon degradation experiment, whole-genome sequencing was performed on *Malikia spinosa* strain AB6 isolated from aerobic benzene degradation enrichment. Bacterial strains were isolated from 5th-week samples of enrichment cultures by conventional methods.

We investigated the diversity of functional genes that play a key role in the degradation of hydrocarbons in enrichment cultures and their presence in pure cultures. Subfamily I.2.C-type C23O genes were PCR amplified by using the primers XYLE3f and XYLE3r developed by Táncsics et al. (2013). In the case of enrichment cultures for the investigation of the degradation of aliphatic hydrocarbons alkB genes were PCR amplified by using the primers alkB 1f_deg and alkB 1r_deg (Kloos et al. 2006, Pérez-de-Mora et al. 2011).

Terminal restriction fragment polymorphism (T-RFLP) fingerprinting was employed to monitor the changes in microbial community composition and the diversity of the functional genes. For digesting PCR products, the following restriction enzymes were used: the 16S rDNA segments were digested with *RsaI* (GT↓AC) restriction enzyme (Thermo Fisher Scientific Inc.); the I.2.C-type C23O gene segments were digested with *AluI* (AG↓CT) restriction enzyme (Thermo Fisher Scientific Inc.); and the *alkB* gene segments were digested with HPyCH4V (TG↓CA) restriction enzyme (New England BioLabs Ltd.). To identify the T-RFs on T-RFLP electropherograms of C23O and *alkB* genes, we created clone libraries for separating common PCR products into individual units. We prepared dendrograms of 16S rDNA and functional gene-based trees by Paleontological statistics software package (Hammer et al. 2001), using Bray-Curtis and Jaccard similarity indexes.

2.2. Conditions of cultivation experiments

Before the enrichment experiments, discussed in my dissertation, we attempted to apply various media to cultivate as many species as possible from the enrichment cultures. The groundwater samples investigated during this experiment came from a previously analysed hydrocarbon-contaminated site (Táncsics et al., 2012, Táncsics et al., 2013, Táncsics et al., 2018). For the cultivation, agar-agar or gellan gum-solidified plates were used. As a carbon source, acetate or malic acid was added to the media, but according to our experience, they did not increase the diversity of colonies developed on the plates. Conventional R2A agar plate was the most effective medium to cultivate bacterial strains; thus, it was selected for strain isolation from enrichments. The most important outcome of these investigations was the successful identification of a novel bacterial strain that belongs to the genus *Sphingobium* within the class *Alphaproteobacteria*. The novel strain was described as *Sphingobium aquiterrae* (Révész et al. 2018).

2.3. Isolation of hydrocarbon-degrading bacteria

During our research, our main goal was to enrich and isolate strains that are capable to degrade hydrocarbons. It makes possible to investigate their metabolic capabilities and potential use for bioremediation purposes. To validate that the strain we isolated is a novel species, many tests had to be carried out. It is necessary to determine phenotypic properties as well as carry out molecular biological and chemotaxonomic studies. We made these investigations according to the suggestions by J. B. Tindall et al. (2010).

3. Results

3.1. Effect of oxygen limitation on the enrichment of bacteria degrading either benzene or toluene

The primary aims of this present study were to evaluate the effect of oxygen limitation on the bacterial community structure of enrichment cultures degrading either benzene or toluene, and to identify which type of I.2.C C23O genes play a key role in microaerobic benzene and toluene degradation. Based on the results of 16S rDNA amplicon sequencing, it was found that members of the class Gammaproteobacteria overwhelmingly dominated the bacterial community of the biofilm material (47% of 16S rDNA sequence reads). Moreover, mainly genera of the Betaproteobacteriales were abundant. Many of these genera contain well-known aromatic hydrocarbon degraders (Prince et al. 2018) or are frequently found in petroleum hydrocarbon-contaminated subsurface environments (Sperfeld et al. 2018). Due to this enormous diversity of potentially petroleum hydrocarbon-degrading bacteria, the biofilm material was an excellent inoculum for the benzene- and toluene-degrading enrichments.

Concentrations of carbon source were determined in every 24-h interval by headspace GC–MS analysis. At the fifth week, aromatic hydrocarbon degradation processes in the enrichments indicated that highly efficient degrading communities evolved in both the aerobic benzene- and toluene-degrading enrichments. On the other hand, under microaerobic conditions, the level of aromatic hydrocarbon degradation was significantly lower. In the benzene-containing enrichments, no significant degradation was observed.

The bacterial community of the aerobic benzene-degrading enrichment (AB1, Fig. 3A) was dominated by a single genus since more than 94% of 16S rDNA sequence reads could be linked to the genus *Malikia*. Besides, members of the genera *Pseudomonas*, *Acidovorax*, and *Flavobacterium* were detectable with notable abundance (> 0.5%). Although the occurrence of the genus *Malikia* in

BTEX-contaminated subsurface environments has already been reported by some studies (Aburto – Ball 2009, Táncsics et al. 2010), their exact role remained hidden. Nevertheless, *Malikia* isolates with aromatic hydrocarbon-degrading ability have not been reported yet in the literature.

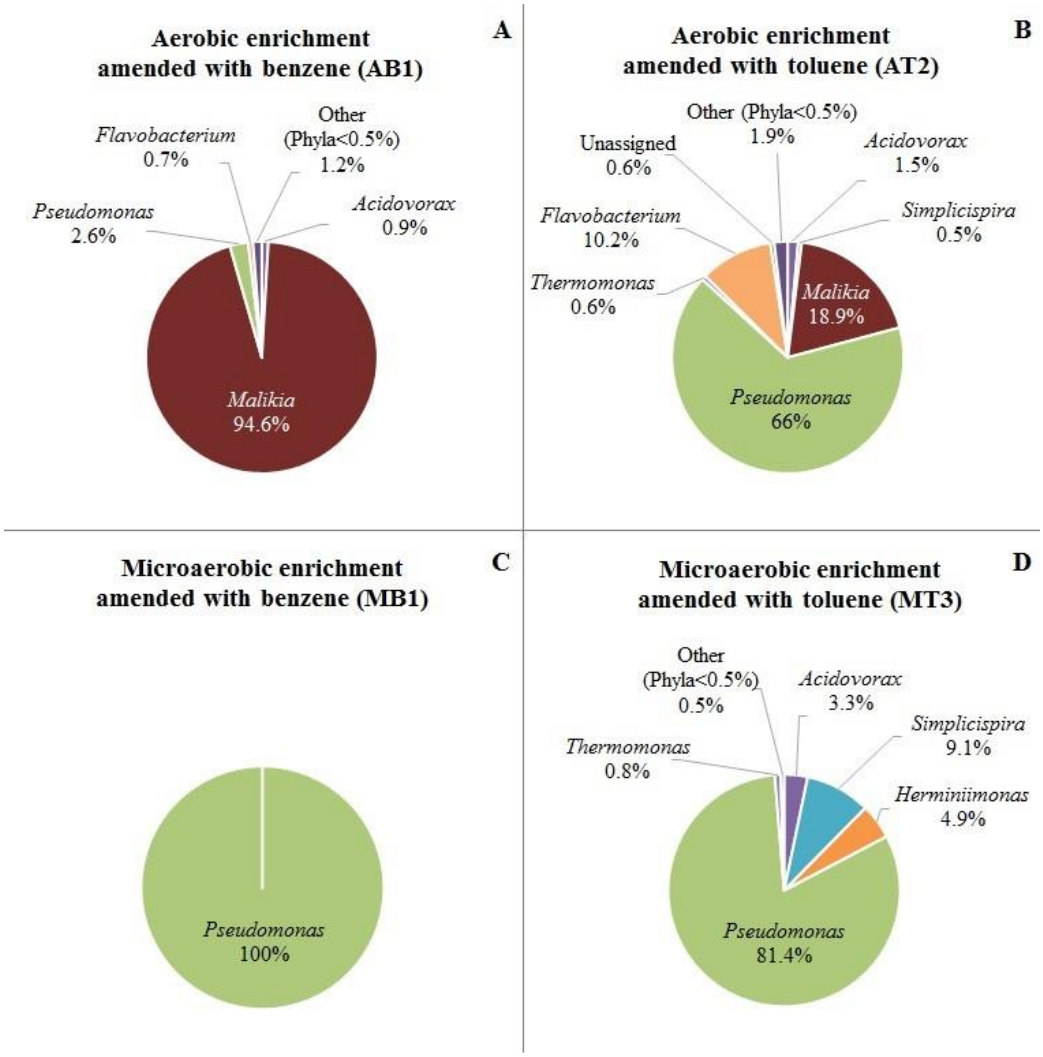

Figure 3: Genus level bacterial community structure of enrichments AB1, AT2, MB1 and MT3 as revealed by Illumina paired-end 16S rDNA amplicon sequencing. All taxa contributing more than 0.5% abundance were depicted.

The bacterial community of the aerobic toluene-degrading enrichment (AT2, Fig. 3B) was dominated by members of the genus *Pseudomonas* (66%) followed by

Malikia (18.9%), *Flavobacterium* (10.2%) and *Acidovorax* (1.5%). The dominant role of *Pseudomonas*-related bacteria in toluene degradation under fully aerobic conditions is well known. The high abundance of sequence reads affiliated with the genus *Malikia* enabled us to assume that these bacteria also played role in the aerobic degradation of toluene.

The bacterial community of the microaerobic, benzene-containing enrichment (MB1, Fig. 3C) was considerably simpler since all of the 16S rDNA sequence reads could be affiliated with the genus *Pseudomonas*. Nevertheless, no significant benzene degradation was observed in this enrichment culture. Presumably, these *Pseudomonas* bacteria were feeding on cell debris or secondary metabolites of other bacteria, which were present in the enrichment only in trace amounts. Contrarily, the bacterial community of the microaerobic, toluene-degrading enrichment (MT3, Fig. 3D) had a bit larger diversity. Although still members of the genus *Pseudomonas* dominated the community (81%), members of the genera *Simplicispira* (9.1%), *Herminiimonas* (4.9%) and *Acidovorax* (3.3%) were also detected with notable abundance.

3.1.1. Diversity and phylogenetic affiliation of subfamily I.2.C-type C23O gene clones in enrichments amended with benzene or toluene

In the aerobic benzene-degrading enrichment culture (AB1), all of the subfamily I.2.C-type C23O gene sequences belonged to a single Operational Phylogenetic Unit (OPU). Moreover, this C23O genotype was identical to that of found earlier by us in the case of aerobic BTEX-degrading enrichment culture and was putatively linked to the genus *Malikia* (Benedek et al. 2018). Surprisingly, this was the only detectable subfamily I.2.C-type C23O genotype in the aerobic toluene-degrading enrichment culture (AT2) as well.

Although no significant benzene degradation was found in the microaerobic enrichments, the largest C23O diversity was detectable in the case of enrichment MB1, since the C23O sequences could be grouped into five OPUs,

which can be linked to unknown bacterial species and *Simplicispira* and *Acidovorax* genera. This result suggests that besides *Pseudomonas*-related bacteria, members of other genera were also present in the community of enrichment MB1, although most probably in trace amounts only. Regarding C23O clone sequences of the microaerobic toluene-degrading enrichment culture MT3, they could be grouped into three OPUs, most of which have been overlapped with sequences shown in microaerobic enrichments amended with benzene.

3.1.2. Description of bacterial strains isolated from enrichment cultures amended with benzene or toluene

Bacterial strains were isolated from the enrichments analysed by Illumina 16S rDNA amplicon sequencing. Altogether 22 strains were isolated representing only four genera. Strains isolated from the aerobic benzene-degrading enrichment AB1 were members of the genera *Pseudomonas*, *Acidovorax* and *Malikia*. The *Malikia*-related isolates were identified as *Malikia spinosa* strains that shows 99.7% and 99.9% 16S rDNA similarity to the type strain *M. spinosa* 83^{T}. Moreover, both of these strains harbored subfamily I.2.C-type C23O gene. For the analysis of aerobic BTEX degradation capability, the *M. spinosa* strain AB6 was chosen. As a result, it was observed that strain AB6 was able to degrade benzene, toluene and ethylbenzene, while it was unable to degrade any isomer of xylene. According to the literature, this was the first time when a *Malikia spinosa* strain could be identified as an aromatic hydrocarbon-degrading bacterium. Strain AB5, which was identified as *Acidovorax delafieldii*, also harbored subfamily I.2.C-type C23O gene. The BTEX-degrading ability of strain AB5 was also investigated, and it turned out that it can degrade benzene only. Strains isolated from the aerobic toluene-degrading enrichment were members of the genera *Pseudomonas* and *Flavobacterium*. Unfortunately, none of the strains harbored subfamily I.2.C-type C23O gene. All of the strains isolated from the microaerobic

enrichments belonged to the *Pseudomonas veronii/extremaustralis* lineage. Although these strains did not harbor subfamily I.2.C-type C23O gene, this lineage of the genus *Pseudomonas* seems to be adapted to microaerobic environments. It was reported that *P. extremaustralis* preferably degrades alkanes under microaerobic conditions (Tribelli et al. 2018).

3.1.3. Whole-genome analysis of Malikia spinosa strain AB6

Since, to date, no publications have been published in which a strain belonging to the genus *Malikia* could have been isolated from an area contaminated with hydrocarbons, it was worth examining the whole genome of the isolated AB6 strain of *Malikia spinosa*. As the whole genome of the type strain (*Malikia spinosa* 83^T) is freely accessible, we have the opportunity to compare it with the *M. spinosa* AB6 strain. The analysis identified 148 proteins that were coded only in the genome of strain AB6 and a notable amount of these proteins could be linked to aromatic compound catabolic processes. Detailed analysis of the genome of strain AB6 revealed that the subfamily I.2.C-type C23O gene was part of a phenol degradation cluster, which coded enzymes of a multicomponent phenol hydroxylase system. Most notably, this cluster was flanked by mobile genetic elements, which fact enables us to presume that this cluster was acquired by strain AB6 through a horizontal gene transfer (HGT) event. Since no other benzene/toluene mono- and/or dioxygenase was found in the genome, it is highly probable that this phenol-degradation cluster is responsible for both the benzene- and the toluene-degrading abilities of strain AB6. Although the ethylbenzene dioxygenase coding gene was not found in the genome of strain AB6, it was able to degrade ethylbenzene. Besides the pathway using the aromatic ring oxidation of ethylbenzene, the ethyl group oxidation catalysed by naphthalene dioxygenase is also feasible (Lee – Gibson 1996, Lee et al. 2019). Screening of the genome sequence for dioxygenases revealed the presence of genes encoding the small and large subunits of a naphthalene dioxygenase, and was part of a naphthalene

degradation gene cluster (i.e. a *nag* operon). This operon encoded salicylate-5-hydroxylase and gentisate 1,2- dioxygenase enzymes as well, which were reported earlier as the key naphthalene metabolic enzymes in the case of *Polaromonas naphthalenivorans* CJ2 and *Ralstonia* sp. U2 (Park et al. 2007; Pumphrey – Madsen 2007, Zhou et al. 2001). Thereby, it can be postulated that strain AB6 could degrade ethylbenzene due to the presence of the nag operon, and it probably has the ability to degrade naphthalene as well.

<u>Novel scientific result (Thesis I):</u> For the first time, a strain belonging to the genus *Malikia* was isolated from hydrocarbon-contaminated area. Results of the present study shed light on the aromatic hydrocarbon-degrading ability of *Malikia spinosa* strains. This observation also revealed that bacteria, which encode subfamily I.2.C-type extradiol dioxygenase enzyme, will not be automatically able to degrade monoaromatic hydrocarbons under microaerobic conditions.

3.2. Effect of oxygen limitation on the enrichment of bacteria degrading diesel fuel/crude oil mixture

The present study aimed to reveal how different microbial communities evolve in diesel fuel/crude oil-contaminated environments under aerobic and microaerobic conditions and also investigated the diversity of *alkB* genes playing a key role in the degradation of alkanes. Illumina 16S rDNA amplicon sequencing has shown that the new biofilm material contained a microbial community overwhelmingly dominated by Betaproteobacteriales of the Gammaproteobacteria. The most abundant genus was *Sulfuritalea* (16% of total 16S rDNA sequence reads), followed by *Azoarcus* (4.8%), *Acidovorax* (2.6%), *Simplicispira* (0.9%), *Thiobacillus* (0.9%), *Hydrogenophaga* (0.7%), *Thauera* (0.6%), *Zoogloea* (0.6%) and *Rhodoferax* (0.5%).

The microbial community of the aerobic enrichment AER2 was dominated by Betaproteobacteriales (36.5% of sequence reads), see Fig. 4. The most abundant Betaproteobacteriales-related genus was *Polaromonas* (14%) followed by *Acidovorax* (6.7%) and *Janthinobacterium* (4%). Several members of the genus *Polaromonas* have already been reported, which can degrade petroleum hydrocarbons such as benzene and toluene (Sun et al. 2010, Xie et al. 2011). Similarly, members of the genus *Acidovorax* are often reported as dominant members of petroleum hydrocarbon-degrading microbial communities (Popp et al. 2006, Daghio et al. 2015).

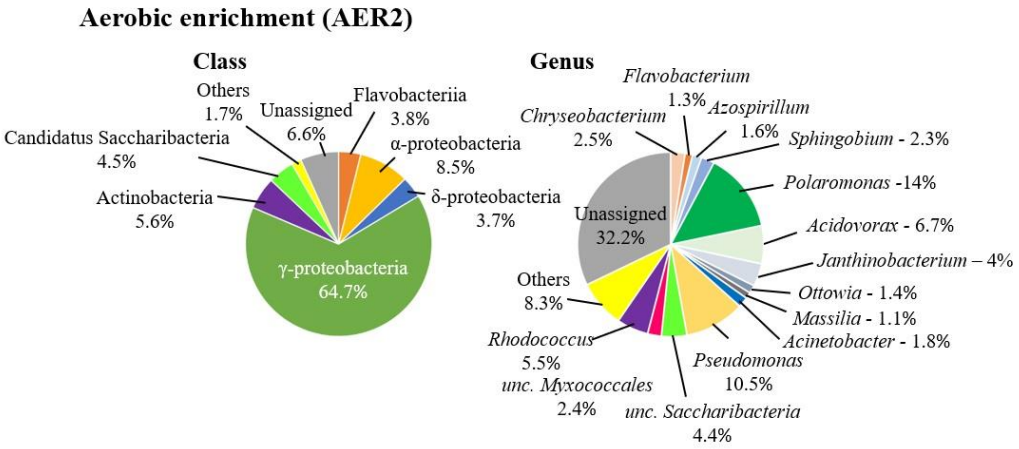

Figure 4: Class and genus level bacterial community structure of the AER2 aerobic enrichment as revealed by Illumina paired-end 16S rDNA amplicon sequencing. All taxa contributing more than 1% abundance were depicted.

At the genus level, *Pseudomonas* proved to be the second most abundant group (10.5%). The role of this genus in petroleum hydrocarbon degradation is well known. Among Alphaproteobacteria, the genus *Sphingobium* (2.3% of sequence reads) has to be noted since several members of this genus are involved in petroleum hydrocarbon degradation under aerobic conditions (Lloyd-Jones – Lau 1997, Pinyakong et al. 2003, Liang – Lloyd-Jones 2010, Révész et al. 2018). Another well-known genus with alkane-degrading species is the *Rhodococcus*

(class of Actinobacteria), whose members were prominent representatives of the aerobic enrichment community. This was expected since *alkB* genes can be found in almost all members of this genus (Táncsics et al. 2015), and especially some species of the so-called "*erythropolis*" clade are highly efficient aerobic alkane degraders.

Interestingly, *Candidatus* Saccharibacteria-related 16S rDNA sequences were also found in relatively high abundance (4.5%). Previously, a certain group (subdivision 3) was found to be abundant in diesel fuel-contaminated soil (Winsley et al. 2014) and SIP studies revealed the possible role of these bacteria in aerobic degradation of toluene and benzene (Luo et al. 2009, Xie et al. 2011).

The microbial community of the microaerobic enrichment MIK1 was considerably simpler than that of the aerobic enrichment. It was overwhelmingly dominated by Gammaproteobacteria (98.8%) as shown in Fig. 5.

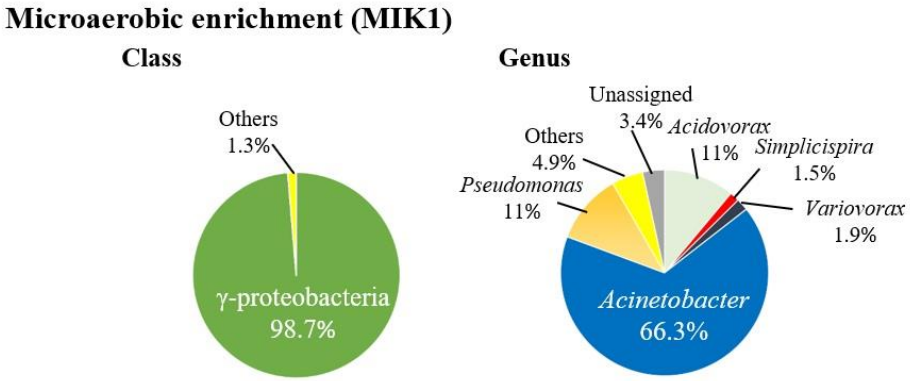

Fig. 5: Class and genus level bacterial community structure of the MIK1 microaerobic enrichment as revealed by Illumina paired-end 16S rDNA amplicon sequencing. All taxa contributing more than 1% abundance were depicted.

At the genus level, *Acinetobacter*-related sequence reads were the most abundant (66.3%). Members of this genus are frequently reported as prominent alkane and aromatic hydrocarbon degraders under aerobic conditions (Lal – Khanna 1996, Di Cello et al. 1997, Margesin et al. 2003, Czarny et al. 2019). *Acinetobacter* species can be considerably abundant in deep subsurface oil reservoirs, and most

probably they are introduced into this environment by injecting water (Orphan et al. 2000, Zhao et al. 2012).

The second most abundant Gammaproteobacterial genus was the *Pseudomonas* (11%). Recently, it has been reported by Tribelli et al. (2018) that in *P. extremaustralis* strain 14–3^{T} expression of genes involved in alkane degradation were up-regulated under low oxygen conditions and the strain was able to use diesel fuel as the sole source of carbon under microaerophilic conditions. Betaproteobacteriales-related sequence reads belonged mainly to the genera *Acidovorax* (11%), *Variovorax* (1.9%) and *Simplicispira* (1.5%). Some members of the genera *Acidovorax* and *Variovorax* are known aromatic hydrocarbon degraders (Sydow et al. 2016, Posman et al. 2017, Singleton et al. 2018). Moreover, *Acidovorax*-related bacteria can be highly abundant in BTEX-degrading enrichment cultures under hypoxic conditions (Benedek et al. 2018).

3.2.1. Diversity of alkB genes in the enrichment cultures amended with diesel fuel/crude oil mixture

The abundance of *alkB* gene T-RFs in triplicates of the enrichment microcosms at the fifth week of the enrichment procedure is shown in Fig. 6. The *alkB* T-RFLP patterns of the parallel microaerobic enrichments were highly similar and could be characterized with the 523-bp long T-RF. In the enrichments AER2 and AER3, the 89-bp long T-RF was the characteristic T-RF, but it was missing in the case of AER1. To link sequence information to the detected T-RFs, *alkB* clone libraries were generated and analysed in case of enrichments AER2 and MIK1.

In the *alkB* clone library of the aerobic enrichment AER2, the sequences could be divided into six OPUs. In case of the largest cluster (63% of clones), sequences showed considerably low similarity to known *alkB* genotypes; therefore, it was not possible to link them to any cultivated bacterium. The most similar nucleotide sequences (with a similarity level of 76–80%) were retrieved

from *Agitococcus lubricus* DSM 5822^{T}, and environmental samples, e.g., crude oil-contaminated seawater (Powell et al. 2010; Wang et al. 2014). T-RFLP analysis of individual clones showed that these sequences could be characterized with the 89-bp long T-RF. Most of the other *alkB* gene sequences were linked to *Pseudomonas* (*P. putida* and *P. chlororaphis* subsp. *aureofaciens*) and *Rhodococcus* genera.

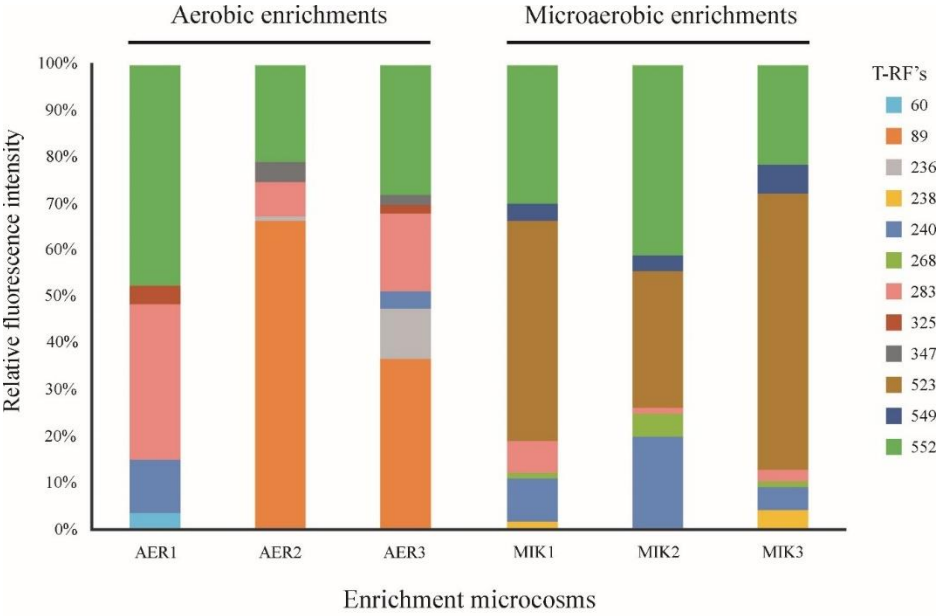

Figure 6: Abundance of *alkB* gene T-RFs in triplicates of the enrichment microcosms at the fifth week of the enrichment procedure.

In the *alkB* clone library of the microaerobic enrichment MIK1, the sequences could be divided into five OPUs. The vast majority of the sequences (71% of clones) are the 523 bp T-RFs. They showed high similarity (98.4% at nucleotide level) with *alkB* gene sequences of *Pseudomonas veronii* strains. The closest relative of *P. veronii* is *P. extremaustralis* that is capable of degrading aliphatic hydrocarbons under microaerophilic conditions (Tribelli et al. 2018). Despite the fact that *Acinetobacter* species were considerably more abundant in enrichment MIK1 than members of the genus *Pseudomonas*, *Acinetobacter*-related *alkB* sequences were found only in low amount (10% of clones, 376-bp T-

RF) and showed the highest similarity (97.8% at nucleotide level) with *alkB* gene of *A. calcoaceticus* strain CA16. However, it has to be noted here that the PCR primers used in the present study for T-RFLP and cloning purposes do not amplify all types of *alkB* genes encoded by *Acinetobacter* species (Jurelevicius et al. 2013). 16% of clone sequences showed high similarity to *Pseudomonas*-related *alkB* sequences (548 and 238-bp T-RF). Some of the *Rhodococcus*-related *alkB* gene sequences were identical with sequences detected in aerobic enrichment cultures.

4.2.3. Phylogenetic affiliation of the abundant alkB genotype by genome binning

Among the *alkB* genotypes recovered in this study, only those remained unaffiliated that belonged to AER-OPU 1 and AER-OPU 5. Since AER-OPU 1 contained the most abundant *alkB* gene sequences in the case of the aerobic enrichment AER2, genome-resolved metagenomics was used to link them to a bacterial lineage. This novel and abundant *alkB* gene was successfully reconstructed in metagenomic assemblies of the aerobic enrichment culture AER2. Almost a total (> 99%) of the genome was assembled successfully. Based on this result, the genome of an unknown Gammaproteobacteria has been obtained; however, no 16S rRNA gene sequence could be linked to the genome. Since no 16S rRNA gene sequence could be binned along with the genome, we investigated the ribosomal proteins of the binned genome. Based on this, the organism is closely related to *Agitococcus lubricus* (Franzmann – Skerman 1981). The bacterium is classified by the National Center for Biotechnology Information (NCBI) as the *Firmicutes* strain; however, based on the whole genome, it actually belongs to the Moraxellaceae family of the class Gammaproteobacteria.

Analysis of the *alkB* gene containing cluster revealed that in the upstream position to the *alkB* gene, an *AraC* family transcriptional regulator coding gene can be found (in opposite orientation). This gene had been designated earlier as

alkR gene in the case of *Acinetobacter* sp. strain ADP1. It was found that this gene also plays a crucial role in alkane degradation. Consequently, the combined metagenome-cultivation approach revealed a novel organism, possibly capable of alkane degradation.

<u>Novel scientific result (Thesis II):</u> Results have shown that members of the genus *Rhodococcus* were abundant members of the enrichment communities only under clear aerobic conditions. On the other hand, microaerobic conditions caused the overwhelming dominance of *Acinetobacter* and *Pseudomonas* genera-related bacteria.

<u>Novel scientific result (Thesis III):</u> A yet unknown but abundant *alkB* genotype was recovered from the aerobic enrichment and was linked to a yet uncultivated member of the family Moraxellaceae by genome binning.

3.3. Describing a new bacterium species *Sphingobium aquiterrae* sp. nov.

During our research a continuous attempt was made to enrich and isolate strains that are capable to degrade hydrocarbons. This provides an opportunity to learn more about their metabolic abilities and their potential use for bioremediation purposes. Strain SKLS-A10^T was isolated from groundwater sample of the 'Siklós' petroleum hydrocarbon contaminated — mainly with benzene, xylene, ethylbenzene and other alkylbenzene compounds — site of Hungary. Strain SKLS-A10^T belongs to the genus *Sphingobium* within the class Alphaproteobacteria.

Since the SKLS-A10^T strain was isolated from groundwater contaminated with BTEX compounds, we investigated which monoaromatic compounds can be degraded by this strain. During the study, BTEX components were individually used as single carbon sources. Accordingly, strain SKLS-A10^T possesses the ability to degrade toluene, *meta*- and *para*-xylene. Tests were carried out following international requirements and the new species was described as *Sphingobium Aquiterrae*.

Novel scientific result (Thesis IV): We isolated a novel *Sphingobium* species, tests required for the description were performed following international requirements. Gas chromatographic-mass spectrometry (GC-MS) confirmed the ability of the strain to completely decompose toluene, *meta*- and *para*-xylene under aerobic conditions. The new species was named as *Sphingobium aquiterrae*.

4. Conclusions, proposals

Numerous studies have shown that the microbial community of hydrocarbon-contaminated media is typically dominated by members of the genera Comamonadaceae and Rhodocyclaceae within the order Betaproteobacteriales (Alfreider et al. 2002; Fahy et al. 2006; Alfreider – Vogt, 2007; Nestler et al. 2007, Martínez-Lavanchy et al. 2015). Functional genes involved in the breakdown of hydrocarbons cannot be linked to cultured bacteria yet, and it was also questionable whether the presence of I.2.C-type C23O genes in the genome can clearly demonstrate the ability of the strain to degrade hydrocarbons under microaerobic conditions. The aim of the present study was also to reveal how different microbial communities evolve in aromatic hydrocarbons or diesel fuel/crude oil-contaminated environments under aerobic and microaerobic conditions.

During our enrichment experiments, it was observed that distinctly different microbial communities take part in the process of hydrocarbon degradation under aerobic and oxygen-limited conditions. The diversity of the investigated functional genes also varied widely according to the availability of dissolved oxygen. During our first experiment, to investigate these questions, aerobic and microaerobic bacterial enrichments amended with benzene or toluene as sole carbon and energy source were established by using biofilm material originated from the same site as in the case of our previous enrichment. In the aerobic benzene-degrading enrichment cultures, the overwhelming dominance of the genus *Malikia* (Betaproteobacteriales) was observed. Two *M. spinosa*-related strains (99.7% and 99.9% 16S rDNA sequence similarity) could be isolated from the samples successfully. Despite the fact that members of the genus are common representatives of the microbial community of areas contaminated with hydrocarbons, according to the literature, this was the first time when a *Malikia spinosa* strain could be identified as an aromatic hydrocarbon-degrading bacterium. It was observed that *M. spinosa* strain AB6 isolated by us was able to

degrade benzene, toluene and ethylbenzene under aerobic conditions. By detailed analysis of the genome of strain AB6, it was possible to examine thoroughly its metabolic abilities and compare its characteristics with the type strain. Comparing the genome sequences of strain AB6 and the type strain *M. spinosa* 83^T revealed that strain AB6 has a ~300 Kbp larger genome than that of the type strain 83^T. This phenomenon is mainly due to the enrichment of genes involved in the degradation of aromatic hydrocarbons. *M. spinosa* strain AB6 harbors the subfamily I.2.C-type C23O gene that is part of a phenol degradation cluster. This cluster was flanked by mobile genetic elements, which fact enables us to presume that this cluster was acquired by strain AB6 through a horizontal gene transfer event. Although the AB6 strain harbors the I.2.C-type C23O gene, *M. spinosa* was only detected in aerobic enrichments. The lack of a gene that can hydroxylate the benzene ring under microaerobic conditions proves that the strain is unable to degrade aromatic hydrocarbons under these conditions. Results of the present study shed light on the aromatic hydrocarbon-degrading ability of *Malikia spinosa* strains, thus providing evidence on our earlier presumption that this bacterium can act as a degrader in BTEX-contaminated environments, but mostly under strictly aerobic conditions (Táncsics et al. 2010, Benedek et al. 2018). This observation also revealed that bacteria, which encode subfamily I.2.C-type extradiol dioxygenase enzyme, will not be automatically able to degrade monoaromatic hydrocarbons under microaerobic conditions. Moreover, based on the whole-genome analysis of *Malikia spinosa* strain AB6, it can be suggested that subfamily I.2.C-type C23O genes can easily spread among members of the Betaproteobacteriales through transposon-mediated HGT events. Under microaerobic conditions, enrichments were amended with either benzene or toluene dominated by *Pseudomonas*-related bacteria. In the microaerobic enrichments, no significant benzene degradation and the slow degradation of toluene was observed. It is assumable that some species of *Pseudomonas veronii/extremaustralis*–related bacteria were successfully adapted to oxygen-limited conditions.

During our second experiment, aerobic and microaerobic bacterial enrichments were set up again. However, diesel fuel/crude oil mixture was used as single carbon and energy source. We investigated the effect of oxygen limitation on the enrichment of bacteria degrading alkanes. We also examined the diversity of alkane-1 monooxygenase genes (*alkB*), which plays a key role in the decomposition of alkanes. Overall, the observed differences between the bacterial community compositions of clear aerobic and microaerobic enrichments can be instructive regarding bioremediation of crude oil/diesel fuel-contaminated environments. Results have shown that members of the genus *Rhodococcus* were abundant members of the enrichment communities only under clear aerobic conditions. This observation is important since rhodococci are frequently used to treat hydrocarbon-contaminated sites due to their enormous metabolic diversity (Kuyukina – Ivshina 2010, Kis et al. 2017). On the other hand, microaerobic conditions caused the overwhelming dominance of *Acinetobacter* and *Pseudomonas* genera-related bacteria. Based on the *alkB* gene diversity analyses, it was also observable that among *Pseudomonas*-related bacteria, the *P. veronii*-lineage was dominant in the microaerobic enrichment cultures. This result together with the fact that the closely related *P. extremaustralis* preferably degrades alkanes under microaerobic conditions enables us to assume that a certain group of *Pseudomonas* species is adapted to these conditions and may have an important role in alkane degradation in subsurface ecosystems. Last but not least, a yet unknown but abundant *alkB* genotype was recovered from the aerobic enrichment and was linked to a yet uncultivated member of the family Moraxellaceae by genome binning. Thus, the present study provides new evidence that the known diversity of alkane-degrading bacteria is still incomplete.

The main goal of our research was to enrich and isolate strains that are capable to degrade hydrocarbons. During our pre-experiment, we successfully isolated a novel *Sphingobium*-related bacterium and confirmed the ability of the strain to completely decompose toluene, *meta*- and *para*-xylene under aerobic conditions. The new species was named as *Sphingobium aquiterrae*.

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