HUNGARIAN UNIVERSITY OF
AGRICULTURE AND LIFE SCIENCES

Theses of doctoral (Ph.D.) dissertation

INVESTIGATION OF MICROBIAL COMMUNITIES CAPABLE OF MICROAEROBIC BENZENE DEGRADATION USING STABILE ISOTOPE METHODS

DOI: 10.54598/006420

Harkainé Bedics Anna

Gödöllő

2025

Ph.D. School

Name: Ph.D. School of Environmental Sciences

Discipline: Environmental Sciences

Leader of School: Csákiné Dr. Michéli Erika, D.Sc.
university professor, head of institute
Hungarian University of Agriculture and Life Sciences
Institute of Environmental Sciences
Department of Soil Sciences and Agrochemistry

Supervisor: Dr. András Táncsics, D.Sc.
university professor, head of department
Hungarian University of Agriculture and Life Sciences
Institute of Environmental Sciences
Institute of Aquaculture and Environmental Safety
Department of Molecular Ecology

…… …….

Approval of School Leader Approval of Supervisors

Table of contents

1. INTRODUCTION AND AIMS

One of the most serious damages to natural ecosystems is caused by oil spills, illustrated by the Deepwater Horizon oil rig disaster (Gulf of Mexico, 2010), which is considered one of the biggest oil spills in history. However, it is not only the seas and oceans but also the land that is affected by oil pollution, e.g. the most significant terrestrial oil spill of the past decade was caused by the Keystone Pipeline leak (Kansas, 2022). In addition, several other historical events attest to the severity of environmental damage caused by oil pollution.

Today, oil pollution from the extraction, transport and processing of oil and the damage it causes to natural aquatic and terrestrial ecosystems are increasing public attention. Hydrocarbon compounds are also irreversibly transforming and degrading the geological environment, surface water and groundwater, and causing significant human health impacts. The extent of oil spills and the damage they cause depend on the physical and chemical properties of the oil constituents and the soil. Certain petroleum derivatives can be toxic and carcinogenic, accumulating in the food chain and passing through microorganisms and primary producers to consumers, including humans.

Increased oil pollution has also led to an increasing focus on its remediation, which is often at the center of scientific studies. In addition to physical and chemical remediation methods, bioremediation activities are also gaining in importance as cost-effective and low environmental impact methods. Indeed, bioremediation uses the hydrocarbon-degrading capacity of microorganisms to restore contaminated sites. Bacterial communities in ecosystems exposed to oil pollution adapt to the new carbon source over time and start to degrade petroleum hydrocarbons. Microorganisms remove the pollutant by different mechanisms, for example, some bacteria have the enzyme system required to degrade petroleum, and genes encoding this enzyme set are spread through the microbial community by horizontal gene transfer, helping them to adapt quickly and efficiently to the new carbon source. The microorganisms work together to degrade a broad spectrum of contaminants, the efficiency of biodegradation is influenced by the availability of alternative electron acceptors, the appropriate electron acceptor and donor ratio, in addition to temperature, pH and available nutrients.

Benzene is also the most harmful of all monoaromatic hydrocarbon compounds for human health and the environment. However, it is also a feedstock for many petrochemical products, and its industrial use is on a huge scale, so we are all exposed to small amounts of benzene every day. Volatile components such as benzene are a major problem in the case of oil spills in the subsurface, and its toxicity is increased by the fact that it is a persistent compound in anoxic environments. It

can also contaminate drinking water supply if dissolved in groundwater in the event of widespread contamination.

Therefore, studying the bacterial community of a petroleum contaminated site, identifying the microorganisms capable of benzene degradation and studying their genomes may lead to the development and application of an effective bioremediation strategy through understanding the genetic background of benzene and other aromatic hydrocarbon degradation.

Regarding the background, the primary aims of our research were as follows:

- The basic question of the research was which bacteria are capable of biodegradation of benzene under microaerobic conditions.
    - In particular, the effect of oxygen limitation and benzene as the sole carbon and energy source on the bacterial community was investigated.
    - Our objectives were to explore how the presence of other BTEX components affects bacterial community development and microaerobic degradation of benzene.
    - Our aim was to identify the bacteria directly involved in the microaerobic biodegradation of benzene using stable isotope probing.
- The research also aimed to isolate new bacterial strains capable of microaerobic degradation of benzene and to perform the necessary characterization to describe a new species.

2. MATERIALS AND METHODS

In order to answer the questions mentioned in our aims, we performed our experiments along two main lines. The groundwater and sediment samples used as the basis for the investigations were taken from Siklós, a damaged site in southwestern Hungary (45′51″25.8°N, 18′17″32.3°E) contaminated with petroleum derivatives.

2.1. Composition of the BTEX-degrading enrichment cultures

In the first experiment, microaerobic enrichment cultures containing only benzene and a mixture of monoaromatic hydrocarbons were established, in which BTEX compounds were the sole carbon and energy source. Enrichment cultures were used to investigate the effect of oxygen limitation and benzene as the sole carbon and energy source on the diversity of the bacterial community. In addition, in the first experiment, we explored the effect of the presence of other aromatic hydrocarbons on the diversity of the bacterial community and how the presence of all BTEX compounds affects the microaerobic degradation of benzene. Details of the composition of the enrichment cultures are summarised in Figure 1.

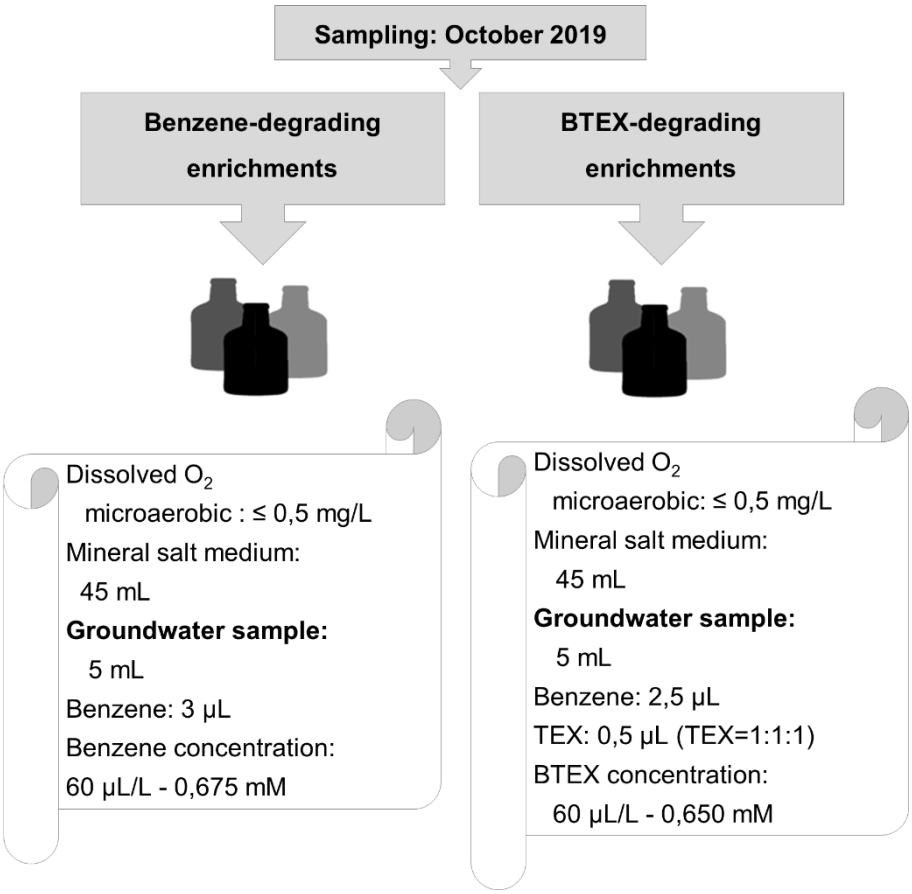


Figure 1: Schematic figure showing the composition of the enrichment cultures.

To establish the enrichment cultures, each serum bottle was filled with 45 mL of mineral salts medium (Fahy et al. 2006), and each enrichment culture also contained 5 mL sample of sedimentary groundwater from Siklós and 3 µL of benzene or a mixture of BTEX compounds (2.5 µL benzene and 0.5 µL other TEX compounds). In both cases, three parallel enrichment cultures were used. Microaerobic (≤ 0.5 mg/L dissolved O_2) conditions were maintained in the bottles. Oxygen consumed by the microorganisms was continuously replenished by injecting sterile air into the bottles. The enrichment cultures were maintained for five weeks with weekly re-inoculation, during this period BTEX was also replenished in the bottles as a carbon source. During the fifth week, the concentration of BTEX compounds was detected every 24 hours with GC-MS measurement.

2.2. Examinations related to the cultured bacterial community

Cultivable bacterial strains were isolated from the fifth week of enrichment cultures using a conventional method. Colonies with different morphologies were inoculated and maintained on R2A plates. To identify the species level of the strains, DNA was isolated from the clean cultures using UltraClean Microbial DNA Kit (Qiagen, Germany) and subsequently amplified using polymerase chain reaction for the 16S rRNA gene and the gene sequence encoding the *C23O* function genes, which were identified by Sanger sequencing. For the strains identified as potential novel species based on 16S rRNA and those possessing the I.2.C *C23O* gene based on PRC, whole genome sequencing was also performed on Illumina NextSeq platform using Seqomics Ltd. For whole-genome phylogenetic analyses, we used the MiGA pipeline (Rodriguez et al. 2018, http1), Genome-Genome Distance Calculator (GGDC) version 2.1 (Meier-Kolthoff et al. 2013, http2) and OAT software (Lee et al. 2016). To determine which BTEX compounds can be degraded based on the genome of the strains, we used the Genoscope MAGE platform (Vallenet et al. 2006, 2009) and the CLC Genomics Workbench Tool v21 (Qiagen, Germany). Subsequently, we tested the BTEX-degrading capacity of the new species by GC-MS (Trace 1300 GC - ISQ Single Quadrupole MS; Thermo Fisher Scientific Inc., USA) with 24 h gas field analysis. Finally, the necessary investigations to describe the new species were carried out.

2.3. Methods used to describe new bacterial species

The isolation of new species capable of degrading BTEX compounds, in particular benzene, has been a major focus of our research, as strains with sufficient degradation potential can be successfully used for bioremediation purposes in the future. For species description, bacterial

strains were characterised using the polyphasic taxonomy method, considering phenotypic, chemotaxonomic, genotypic and phylogenetic aspects of the isolates (Tindall et al. 2010).

2.4. Methods used to investigate the bacterial community

For a comprehensive analysis of the microbial community selectively enriched in the enrichment cultures, we first isolated the genomic DNA of the community from the fifth week of cultures containing an already stable hydrocarbon-degrading bacterial community using the NucleoSpin Soil Kit (Macherey-Nagel GmbH & Co KG, Germany). Subsequently, T-RFLP (terminal restriction fragment length polymorphism) was used to reveal the diversity of the microbial community in the enrichment cultures. The PCR was performed using VIC labeled (Life Technologies™, USA) forward primer, followed by restriction digestion of the products with *Alu*I (AG↓CT) (Thermo Fisher Scientific Inc., USA) enzyme. Based on T-RFLP analysis, representative members were selected from the parallels of the fifth week endpoint samples and 16S rDNA amplicon sequencing was performed. Amplification of the 16S rDNA variable V3 and V4 regions was performed on Illumina MiSeq platform by Seqomics Ltd. (Mórahalom, Hungary). Phylogenetic data analysis was then carried out using MOTHUR software (v1.41.1) (Schloss et al. 2009) following the MiSeq SOP (Standard Operation Procedure) recommendation (Kozich et al. 2013, http3).

2.5. Investigations with stable isotope labelling method

For the second experiment, we used stable isotope labelling method to identify the microorganisms actively involved in microaerobic biodegradation of benzene using microcosm containing benzene labelled on all carbon atoms, and to explore the similarities and differences between aerobic and microaerobic benzene-degrading bacterial communities after one week of incubation.

For the experiment, the microcosms were set up similarly to the enrichment cultures (Figure 2). The serum bottles were supplemented with modified mineral salt medium, and each microcosm also contained 5 g of sediment sample from Siklós (May 2022 sampling) and 5 µL of stable isotopically labelled benzene, or unlabelled benzene for the three parallel controls. Aerobic (7-8 mg/L dissolved O_2) condition was maintained in three parallel bottles and microaerobic ($\leq$ 0.5 mg/L dissolved O_2) condition in the other three. The used air was continuously replenished in the bottles. Microcosms were incubated for one week in a shaking thermostat at 28° at 150 rpm. During the week, benzene concentration was monitored every 24 hours by GC-MS.

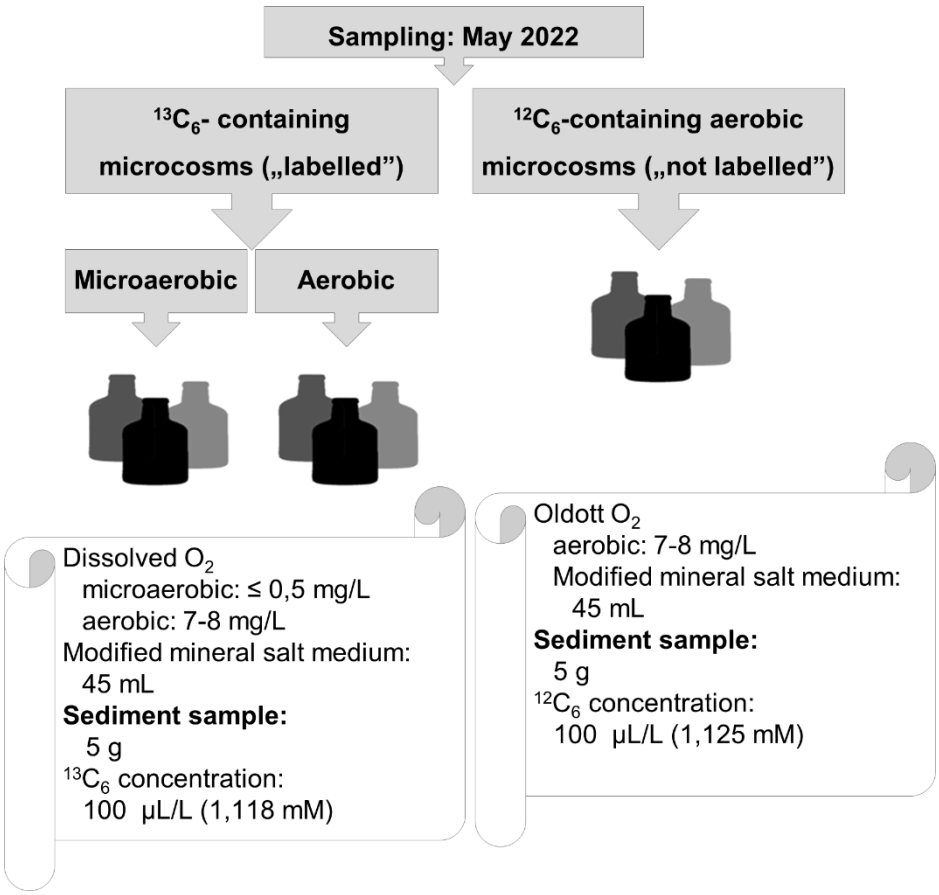


Figure 2: Schematic figure showing the composition of the microcosms

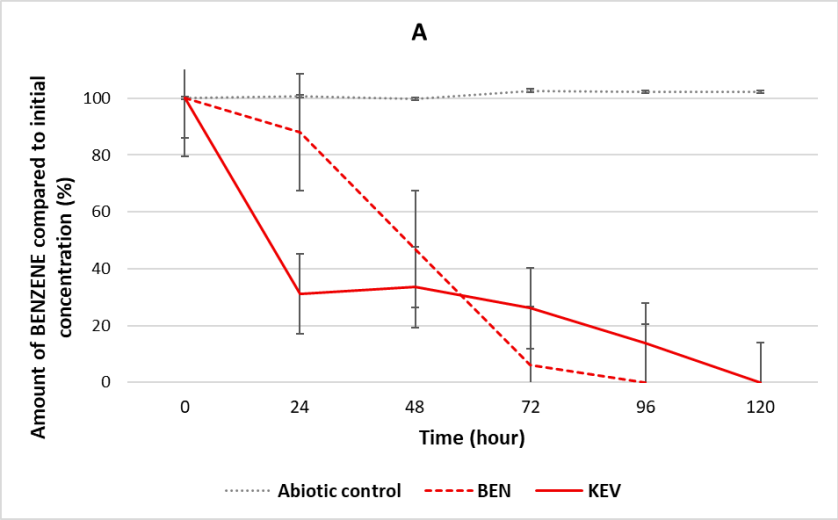
DNA samples extracted from the microcosms were analysed by T-RFLP analysis, then separated by density using caesium chloride gradient ultracentrifugation, and the 16S rDNA copy number of each fraction was determined by qPCR to reveal which fractions contained the labelled and unlabelled DNA. The heavy fraction peaks were detected at a density value of 1.72-1.74 g/mL and the light fraction at 1.66-1.68 g/mL, which is in agreement with the scientific literature (Lueders et al. 2004, Winderl et al. 2010). DNA samples extracted from the original aerobic and microaerobic community prior to fractionation, and from the peaks of the light ($^{12}C_6$) and heavy ($^{13}C_6$) DNA fractions, as well as from the intermediate fractions, were analysed using 16S rDNA Illumina amplicon sequencing to identify the members of the microbial community that are able to metabolize benzene under aerobic and microaerobic conditions.

3. RESULTS AND DISCUSSION

3.1. Evaluation of the results of microaerobic benzene-degrading enrichment cultures

3.2.1. Benzene biodegradation efficiency of enrichment cultures

Figure 3 shows the consumption of benzene in benzene-containing enrichments and BTEX mixture-containing enrichments. Overall, benzene decomposition was more efficient when benzene was present as the sole carbon and energy source in the enrichment cultures, but within 24 hours, benzene degradation was more intense in the mixture enrichment. Figure 3 B shows the decrease in the amount of components in the BTEX mixture-containing enrichments, which allowed us to make conclusions about substrate interactions between the BTEX compounds. In an oxygen-limited environment, toluene and ethylbenzene stimulate the biodegradation of each other and benzene, which is the reason why benzene degradation was found to be more efficient in the first 24 h (Dou et al. 2008). In addition, it was observed that the xylene compounds were also consumed at a higher rate on the first day, which may also be due to the presence of toluene and ethylbenzene, as these compounds could stimulate the degradation of xylene isomers at low concentrations. However, cometabolic degradation of xylenes, especially *p*- and *o*-xylenes, has also been observed in the presence of toluene (Chang et al. 1993, Oh et al. 1994, Littlejohns et al. 2008), as due to the similarity of the two compounds, their degradation pathways overlap (Evans et al. 1992, Tsao et al. 1998). In our experiment, the benzene and xylene isomers are assumed to have inhibited each other's biodegradation, which explains the decreasing trend in the degradation of benzene, *p*-, *m*-, and *o*-xylene compounds after the first 24 hours, and why it took another 96 hours for them to completely disappear from the bottles.



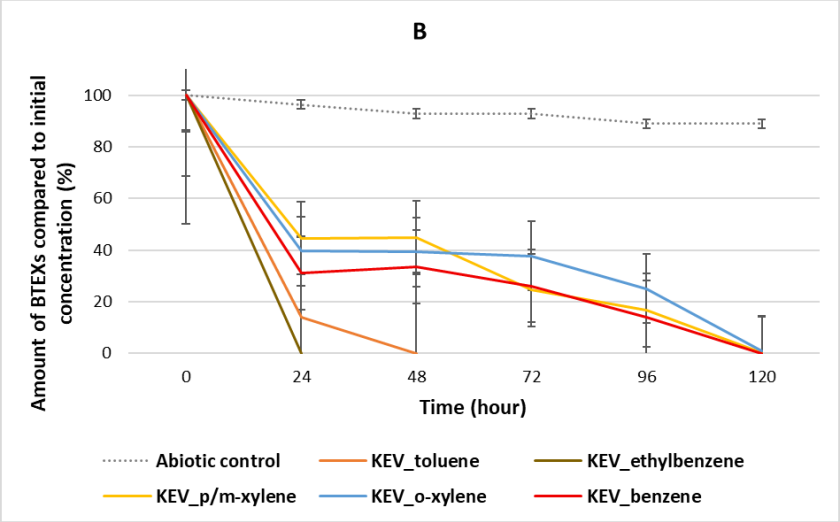


Figure 3: Monitoring the BTEX degradation of the fifth week of enrichment cultures. The amount of BTEX compounds is plotted as % of the initial concentration (GC-MS measurement at hour 0). **A**- Comparison of microaerobic benzene degradation in enrichment cultures containing benzene (BEN) and BTEX mixture (KEV); **B**- Degradation of aromatic hydrocarbon components in enrichment cultures containing BTEX mixture (KEV). The abiotic control in Figure (B) is the average of all BTEX compounds measured every 24 h (at a total concentration of 60 µL/L).

3.2.2. Investigation of bacterial communities in microaerobic enrichment cultures by 16S rDNA amplicon sequencing

The bacterial communities of the enrichment cultures containing benzene and other BTEX compounds were similar in composition, but there were significant differences in the proportion of dominant species (Figure 4). While the benzene-degrading cultures were dominated by members of the genus *Rhodoferax*, the BTEX-degrading cultures were dominated by the genus *Pseudomonas*. In addition, the genus *Acidovorax* was also abundant and was present in similar abundance in both types of enrichment cultures. In the BEN2 sample, the genus *Acidovorax* (typical of enrichment cultures containing only benzene), while in the KEV1 samples, the genus *Pseudomonas* (dominant in BTEX-containing enrichment cultures) showed much more intensive growth, which could be due to the inhomogeneity of the initial sedimentary groundwater sample, or it could be possible that these species were more successful than others in the competition for carbon sources in one of three samples. In addition to the most significant genera, the *Geothrix* and *Brevundimonas* were identified from the BTEX-containing enrichments, and the genera *Rhizobium*, *Azoarcus*, *Pseudoxanthomonas* and *Xanthobacter* were detected from the benzene-degrading cultures.

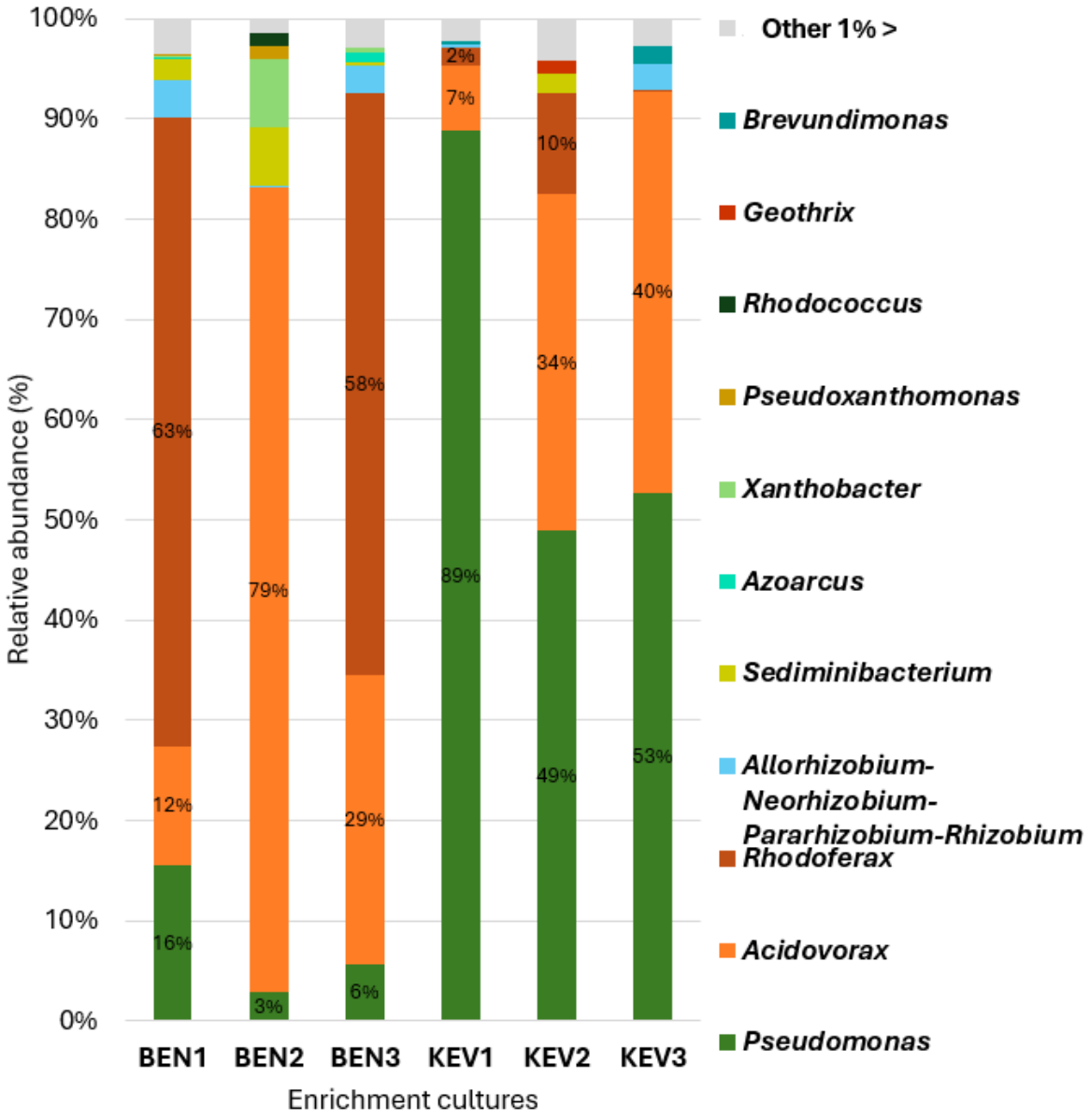


Figure 4: Genus-level distribution of bacterial communities in BEN and KEV enrichment cultures according to data of the 16S rRNA gene in Illumina amplicon sequencing. Only taxa with abundance greater than 1% are shown.

3.2.3. The composition of the cultivable bacterial community isolated from the enrichment cultures

Bacterial strains were also isolated from the fifth week enrichment cultures. The conventional culture procedure was used to collect a diverse bacterial community of ten different genera among the 26 strains isolated from the enrichment cultures (Figure 5). The only dominant community members isolated from both types of enrichment cultures were those belonging to the genus

Pseudomonas. The two most important members of the cultivated bacterial community were isolated from the benzene-degrading enrichment culture. One of them belonged to the genus *Pinisolibacter* showed 97.4% similarity to *P. ravus* strain E9^T based on the 16S rRNA gene, suggesting that the strain could be described as a new species of the genus *Pinisolibacter*, which was successfully isolated for the first time from a subsurface environment contaminated with aromatic hydrocarbons. Our other strain was most closely related to *Ideonella dechloratans* strain CCUG 30977^T, with a similarity of 99.15% based on the 16S rRNA gene and according to the I.2.C. type *C23O* gene specific PCR results it possesses this functional gene, suggesting that it is a potentially new species of the genus *Ideonella*.

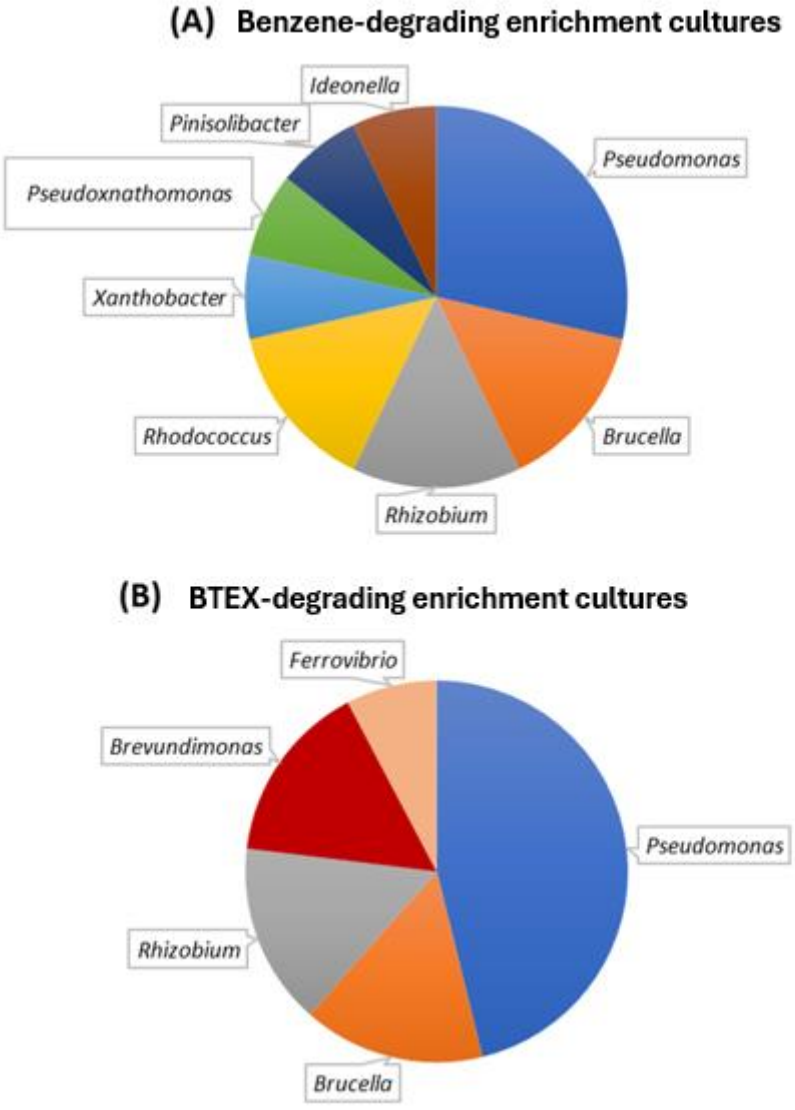


Figure 5: Genus level representation of bacterial strains isolated from enrichment cultures. **A** - Cultivated bacterial community from benzene-degrading enrichment cultures; **B** - Cultivated bacterial community from enrichment cultures containing BTEXs

3.2. Evaluation of the results of stable isotope probing (SIP) of benzene-degrading microcosms

3.2.1. Investigation of aerobic and microaerobic benzene-degrading microbial communities by 16S rDNA amplicon sequencing

The aerobic microcosms were dominated by members of the genus *Pseudomonas* with 40-51% of relative abundance and in addition members of the genera *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (10-15%) and *Thauera* (12-15%) were present in high abundance. The major *Pseudomonas* OTUs were associated with members of the *P. aromaticivorans* (35-45%) and *P. stutzeri* species complexes (4-5%) with 99.4% of 16S rRNA gene sequence similarity, which are major aromatic hydrocarbon-degrading microorganisms (Banerjee et al. 2022, Brown et al. 2017, Li et al. 2022). Members of the genera *Zoogloea* (2-3%), *Sulfuritalea* (~2%) and *Malikia* (~1.5%) were present as less significant community members in all aerobic microcosms.

The microaerobic microcosms were dominated by members of the genera *Malikia* (26-40%) and *Azovibrio* (20-28%). It was observed that the abundance of the genus Pseudomonas was significantly reduced compared to aerobic microcosms, which may be due to the reduction of *P. aromaticivorans* OTU to around 1% compared to 35-45% in aerobic microcosms. The genus *Azovibrio* was detected only in microaerobic microcosms and was not detected in aerobic experiments. Little is known about the ecological role of the genus *Azovibrio* and its hydrocarbon-degrading capacity. Táncsics et al. (2023) successfully identified *A. restrictus* in a microaerobic xylene-degrading enrichment culture in which it was assumed to occupy a similar ecological niche as the genus *Rhodoferax*. Prior to this, the function of *Azovibrio* in BTEX degradation had never been reported, as the ecological role of this bacterium is often hidden even in relevant environmental microbiological studies. The closest relative of the genus *Azovibrio* OTU was also *A. restrictus* with only 95.7% 16S rRNA gene sequence similarity. Based on Táncsics et al. (2024), this bacterium belonging to the genus *Azovibrio* isolated from the damage site is assumed to use the *meta*-cleavage pathway to degrade benzene. The genus-level distribution of the bacterial community of stable isotopically labelled benzene-degrading microcosms (BENSIP) is summarized in Figure 6.

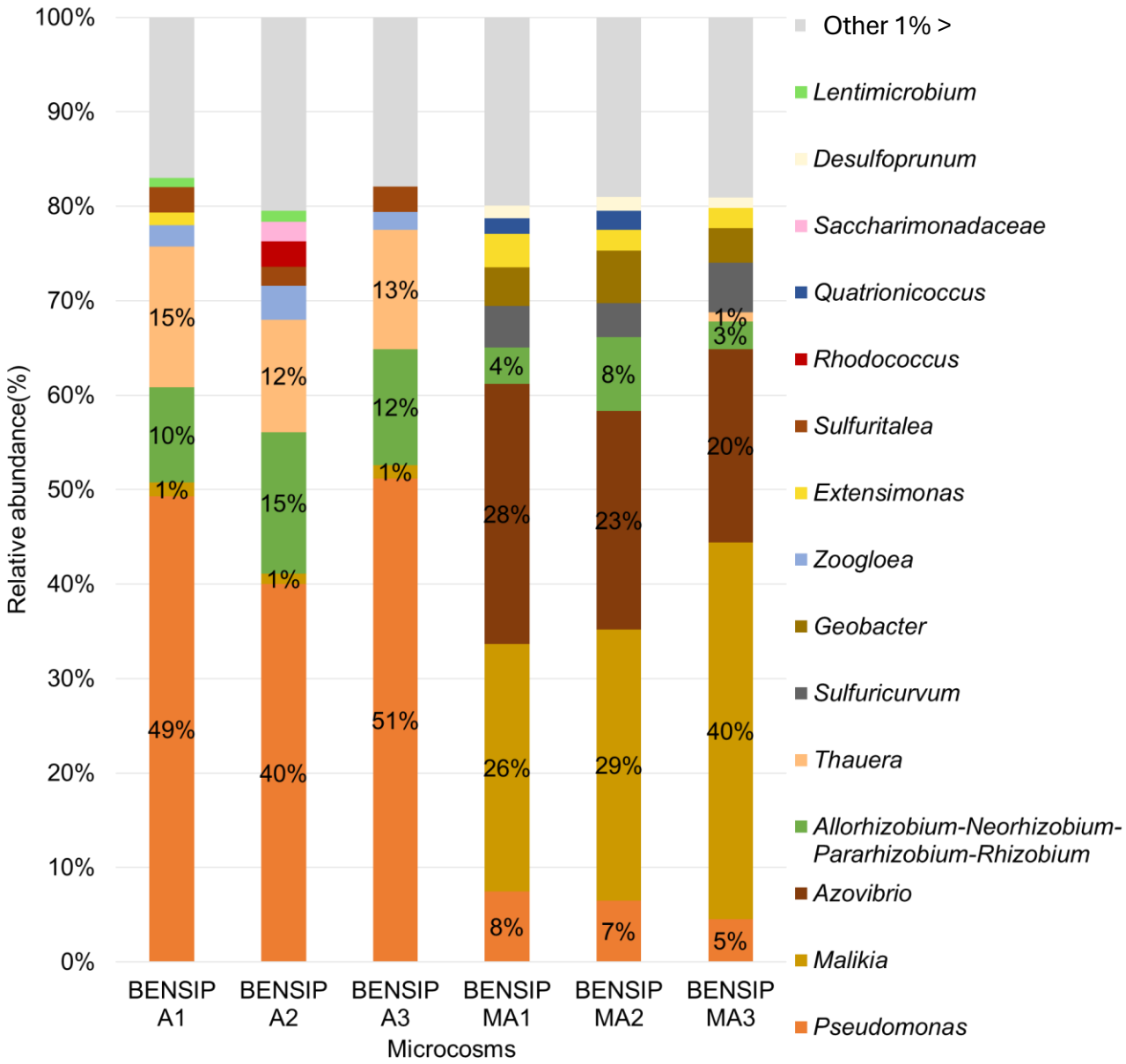


Figure 6: Genus-level distribution of the bacterial community of stable isotopically-labelled benzene-degrading microcosms (BENSIP) according to Illumina 16S rRNA gene amplicon sequencing data. Only taxa with abundance greater than 1% are shown. A- aerobic; MA- microaerobic.

3.2.2. Evolution of microbial communities in light and heavy DNA fractions

After separating the DNA samples by density using ultracentrifugation, the 16S rDNA copy number of each fraction was determined by qPCR, deducing which fractions contain the labelled (heavy fraction: A7, MA6) and unlabelled (light fraction: A10-A14, MA11-MA14) DNA, and intermediate fractions (A8, MA7-MA10) were determined based on the copy number of 16S rDNA and density values. The heavy fraction contains DNA from members of the community that have integrated the isotopically labelled benzene into the DNA chain during metabolism. Figure 7

shows the qPCR results of aerobic control containing unlabelled benzene and of fractions separated from aerobic and microaerobic microcosms containing isotopically labelled benzene.

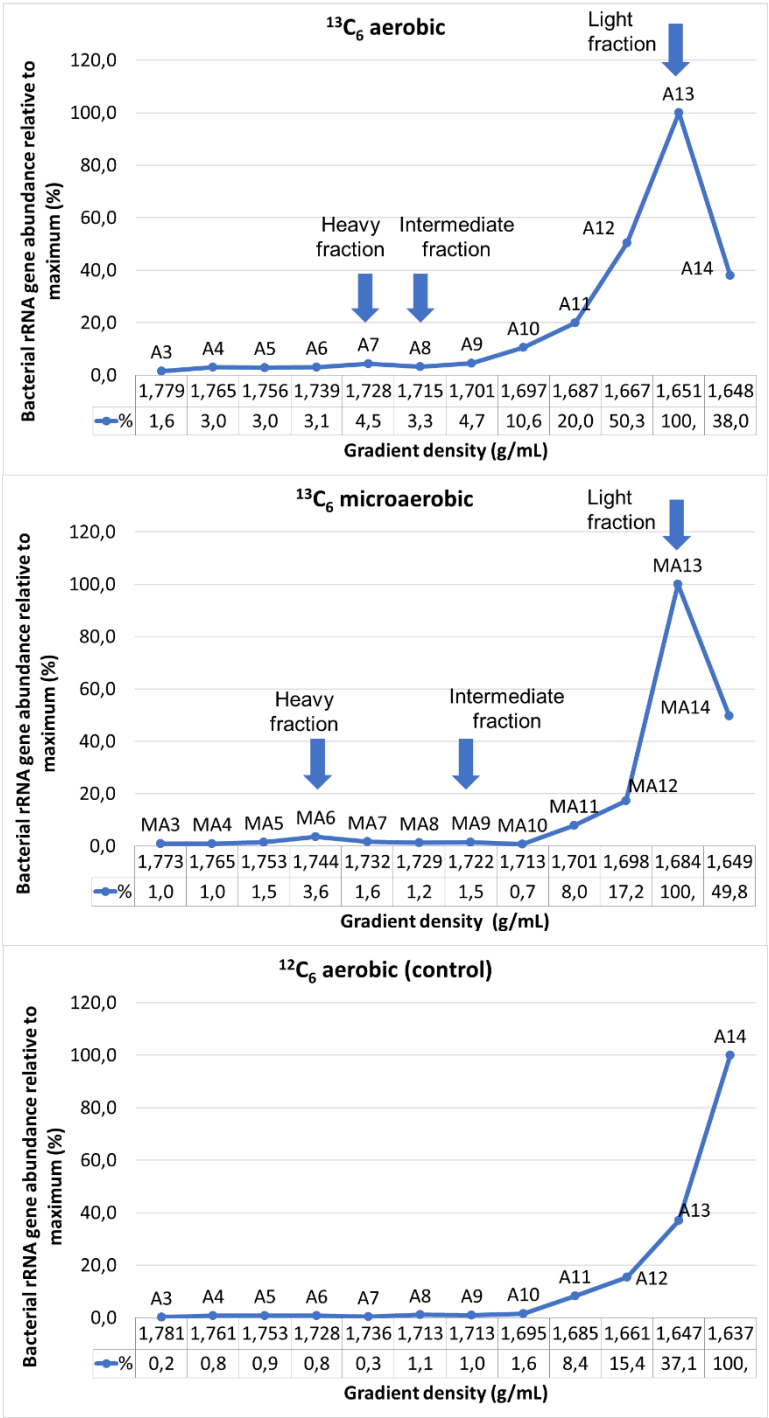


Figure 7: Separation of fractions by density and determination of the light and heavy DNA peaks based on 16S rRNA gene abundance. The fractions (A-aerobic fractions, MA-microaerobic fractions) are numbered from 3 to 14 (fractions 1-2 and 15-18 were excluded from further analysis). The table below the x-axis shows the gradient density values (g/mL) assigned to each fraction at the top and the percentage values of 16S rRNA gene abundance at the bottom.

After the determination of the light and heavy DNA peaks, 16S rDNA amplicons sequencing was performed from their fractions (MA6, MA13, A7, A13) and from the selected intermediate DNA fractions (A8, MA9), which results are shown in Figure 8.

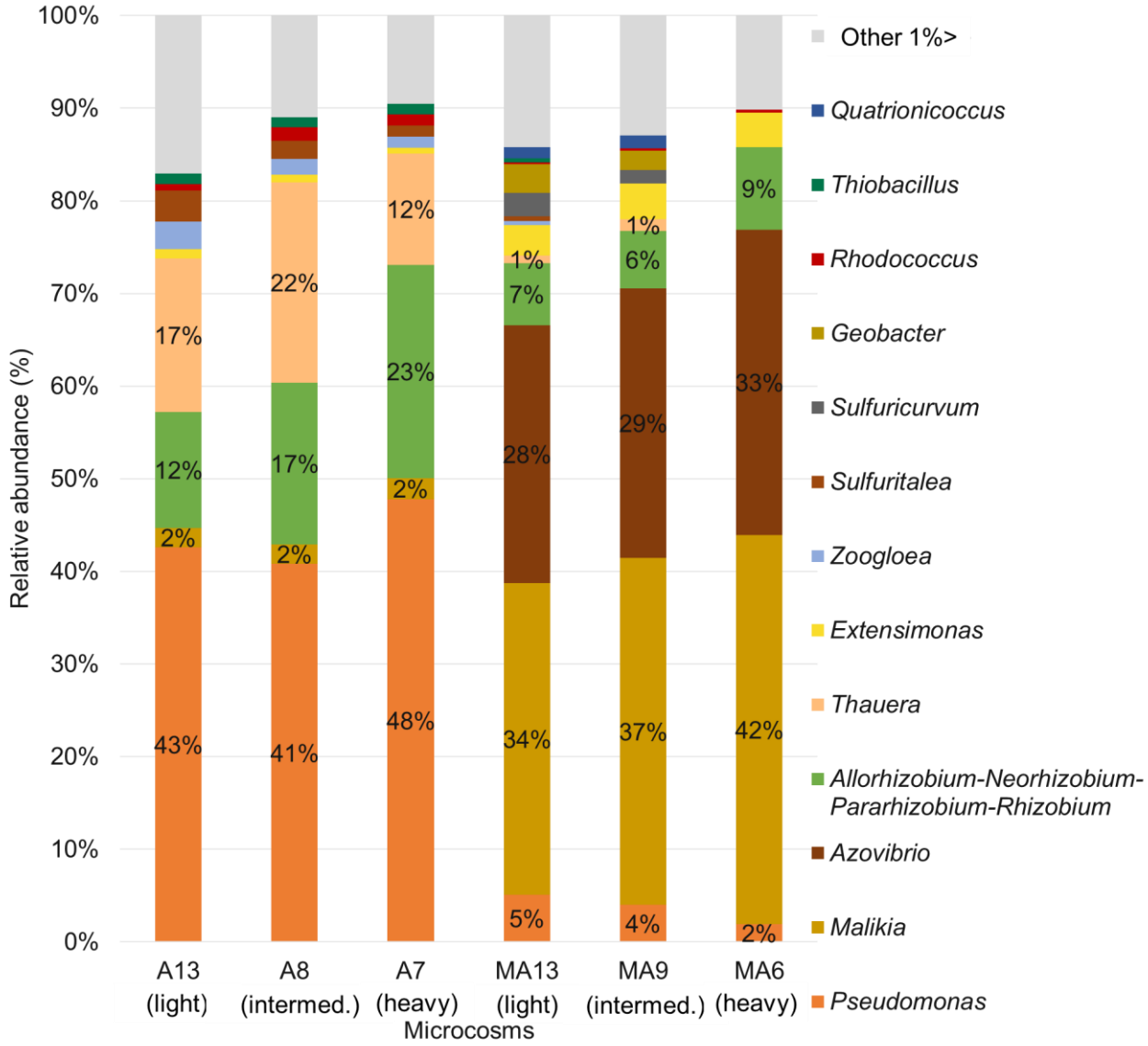


Figure 8: Genus-level distribution of the bacterial community of light and heavy DNA fractions separated from stable isotopically labelled benzene-degrading microcosms according to Illumina 16S rRNA gene amplicon sequencing data. Only taxa with abundance greater than 1% are shown. A- aerobic; MA- microaerobic.

In aerobic microcosms, the genus *Pseudomonas* and *Rhizobium* were identified as benzene degraders, as they were present in the heavy DNA fraction with increased abundance (*Pseudomonas*: 48%, *Rhizobium*: 23%) compared to the light fraction (*Pseudomonas*: 43%, *Rhizobium*: 12%), however, in order to draw a clear conclusion on the role of the genus

Pseudomonas in aerobic benzene degradation, it is necessary to examine the species level of this genus to determine which species are responsible for the quantitative increase. For *P. aromaticivorans* OTU, the 37% abundance observed in the light fraction increased to 43% in the heavy DNA fraction, but for *P. stutzeri* OTU the opposite trend was observed, as a higher abundance was detected in the light fraction (4% and 2.7%). The results obtained clearly indicate that *P. aromaticivorans* is a prominent benzene degrading bacterial species under aerobic conditions. Another interesting observation was that the abundance of bacteria belonging to the genus *Thauera* was highest in the intermediate fraction (22%). The exact explanation of this phenomenon is not known, but it can be assumed that the generation time of these bacteria is longer than the others and the heavy isotope is less enriched in their DNA, despite their ability to degrade benzene. In the case of microaerobic microcosms, the two main community members, *Malikia* and *Azovibrio*, can be clearly identified as benzene degraders, as both genera showed significantly higher abundances in the heavy fraction (*Malikia* - 42%, *Azovibrio* - 33%) compared to the light fraction (*Malikia* - 34%, *Azovibrio* - 28%). For microaerobic microcosms, no significant enrichment was observed for any taxon in the selected MA9 intermediate fraction, it functioned as a real intermediate fraction.

3.3. Description of new aromatic hydrocarbon-degrading bacterial species

3.3.1. Characterization and description of Pinisolibacter aquiterrae B13^T as a new species

The genus *Pinisolibacter*, belonging to the family *Ancalomicrobiaceae* (*Hyphomicrobiales*; synonym *Rhizobiales*), was recently described by Dahal and colleagues (2018b), and its role in natural communities in oil-contaminated areas has not been previously reported. As only the second member of its genus, *Pinisolibacter aquiterrae* is described not only through the characterisation of *Pinisolibacter* sp. strain B13T, but also through experiments on two isolates of the same species. In addition to strain B13^T, the other strain, *Pinisolibacter* sp.MA2-2, was isolated by Dr. Sinchan Banerjee from a microaerobic enrichment culture of a mixture of xylene isomers in the same ratio, for which the sample was collected in October 2019 from Siklós.

Comparative analysis of the 16S rRNA gene sequences showed that strains B13^T and MA2-2 are most closely related to *Pinisolibacter ravus* E9^T (97.4%), *Siculibacillus lacustris* SA-279^T (96.3%) and *Ancalomicrobium adetum* NBRC 102456^T (94.05%), which clustered on a lineage.

Whole-genome analysis of strain B13^T shows that it has the genetic background required for the degradation of monoaromatic hydrocarbons. The I.2.B type catechol-2,3-dioxygenase (*C23O*) gene, which is specific for alphaproteobacteria, was identified as part of a gene cluster encoding a partial *meta*-cleavage pathway. The gene cluster we identified encoding the *meta*-cleavage

pathway was incomplete because the genes required for ring activation were missing, only genes required for ring cleavage and catalysis of the downstream steps of the pathway were found. However, a gene cluster encoding a toluene monooxygenase capable of activating the aromatic ring was found in the genome, which could potentially complement the gene cluster encoding the partial *meta*-cleavage pathway and provide aromatic hydrocarbon degradation capacity for strain B13^T. The gene cluster encoding catechol-2,3-dioxygenase and toluene monooxygenase was also found in strain MA2-2, which was fully identical to that identified in the B13^T type strain. In addition, the gene encoding ethylbenzene dehydrogenase, a possible initial enzyme in ethylbenzene metabolism, was also present in the genomes of strain B13^T and MA2-2.

The ability of *Pinisolibacter* sp. strains B13T and MA2-2 to degrade aromatic hydrocarbons was also confirmed by GC-MS analysis, which showed that among the six aromatic hydrocarbons, strains B13^T and MA2-2 were able to utilize only ethylbenzene as the sole carbon source. However, in experiments in the presence of all BTEX compounds, we found that all aromatic hydrocarbons could be degraded, presumably by stimulating effect of the aromatic compounds on each other's degradation efficiency and by activating cometabolic processes.

The investigations necessary for the description of the species were carried out following the polyphasic taxonomy approach in accordance with international requirements. Based on the results of phylogenetic, morphological, biochemical and chemotaxonomic analyses of *Pinisolibacter* strain B13^T, strain B13^T represents a new species within the genus *Pinisolibacter*, which we named *Pinisolibacter aquiterrae*. The type strain has been deposited in the strain collections with the accession numbers LMG 32346^T and NCAIM B.02665^T.

3.3.2. Characterisation and description of Ideonella benzenivorans strain B7^T as a new species

Strain B7^T is a member of the genus *Ideonella* within the family *Comamonadaceae*, which includes most of the BTEX-degrading genera. Based on whole-genome phylogenetic analyses, it was most closely related to *Ideonella dechloratans* CCUG 30898^T and it represents a separate lineage within the genus *Ideonella*.

A whole genome analysis of strain B7^T was performed to determine the genetic background required for the degradation of monoaromatic hydrocarbons and the catechol-2,3-dioxygenase (*C23O*) was identified in a gene cluster responsible for phenol degradation, encoding a multicomponent phenol hydroxylase (mPH) and a complete *meta*-cleavage pathway. In addition, a gene responsible for encoding a subunit of ethylbenzene dioxygenase was also identified, suggesting that the B7^T strain may also be involved in ethylbenzene metabolism. However, the

xylene monooxygenase genes catalysing the formation of xylenes to methylbenzyl alcohol were missing, so the genetic background of the strain for xylene degradation was incomplete.

The aromatic hydrocarbon degradation capacity of strain B7^T was also confirmed by GC-MS measurements, where we observed that strain B7^T was able to degrade benzene, toluene and ethylbenzene as sole carbon and energy source both aerobically and microaerobically. The B7^T strain was not able to utilize xylenes as sole carbon and energy sources, however, in the presence of all BTEX components, the amount of all xylene isomers decreased within 48 hours. However, after benzene, toluene and ethylbenzene were completely degraded from their environment, the xylenes did not decrease further, due to the fact that under aerobic conditions in the presence of benzene and toluene the xylene compounds are cometabolized, but in their absence the strain was not able to completely degrade the xylene compounds as sole carbon source.

The investigations necessary for the description of the species were carried out following the approach of polyphasic taxonomy in accordance with international requirements. Based on the results of phylogenetic, morphological, biochemical and chemotaxonomic analyses of *Ideonella* strain B7^T, it was concluded that strain B7^T represents a new species within the genus *Ideonella*, which was named *Ideonella benzenivorans*. The type strain has been deposited in the strain collections with accession numbers LMG 32345^T and NCAIM B.02664^T.

3.3.3. Characterization and description of Acidovorax benzenivorans strain D2M1^T as a new species

The characterization of a strain of *Acidovorax* sp. isolated from a *meta*-xylolytic enrichment culture by Banerjee et al. (2022) was also carried out within this thesis. This strain is most closely related to *Acidovorax delafieldii* strain DSM64^T based on the 16S rRNA gene (99.93%), as well as to the OTU representing the genus *Acidovorax* in benzene-containing (BEN) enrichment cultures. These results suggest that it is an endemic, dominant community-forming species of the damage site, potentially involved in microaerobic benzene degradation, and the results of genome analysis and description as a new species are presented within this thesis. Based on whole genome phylogenetic analyses, strain D2M1^T can be described as a new species within the genus *Acidovorax*.

During the genome analysis, we identified the I.2.C-type catechol-2,3-dioxygenase (*C23O*) gene as part of a phenol-degrading gene cluster encoding a complete *meta*-cleavage pathway, in which the aromatic ring is activated by the multicomponent phenol hydroxylase (mPH) system. Three transposon-encoding ORFs belonging to the IS3 and IS5 families bordering the cluster were also identified in the downstream position. Such mobile genetic elements mediate horizontal gene

transfer, providing rapid adaptation of microorganisms to new contaminants and increasing the diversity of *C23O* genes (Díaz 2004). The presence of transposons near the phenol-degrading gene cluster presumably indicates that strain D2M1^T has acquired the gene cluster through horizontal gene transfer. In addition to the extradiol cleavage pathway, whole genome analysis of the *Acidovorax* strain revealed the presence of the chlorocatechol 1,2-dioxygenase gene responsible for the intradiol cleavage of the aromatic ring, but no other genes of the *ortho*-cleavage pathway were identified close to this gene. Based on these results, it was suggested that the genes of the *meta*-cleavage pathway are expressed due to the presence of BTEX compounds and the aromatic hydrocarbons are degraded through this pathway. Another possible pathway for ethylbenzene degradation is through the enzymes required to degrade naphthalene (Lee and Gibson 1996, Lee et al. 2019), and we found evidence of the occurrence of this gene in the genome, suggesting that strain D2M1^T may also be capable of ethylbenzene degradation.

The ability of strain *Acidovorax* sp. D2M1^T to degrade aromatic hydrocarbons was verified by GC-MS, showing that strain D2M1^T was able to utilize benzene and ethylbenzene as sole carbon and energy sources aerobically and microaerobically, while it was not able to degrade xylenes. In the presence of other monoaromatic hydrocarbons, toluene also degraded within 5 days as a result of a very slow process, similar to benzene. Under oxygen-limited conditions, toluene and ethylbenzene are both increasing the biodegradation of each other and of benzene, but under aerobic conditions they inhibit benzene biodegradation. However, the aerobic degradation of toluene is enhanced by the presence of benzene (Littlejohns and Daugulis 2008). Together, these two factors may explain the degradation of toluene and the almost identical rate of degradation of the two compounds.

The investigations necessary for the description of the species were carried out following the approach of polyphasic taxonomy in accordance with international requirements. Based on the results of phylogenetic, morphological, biochemical and chemotaxonomic analyses of *Acidovorax* strain D2M1^T, it was concluded that strain D2M1^T represents a new species within the genus *Acidovorax,* which was named *Acidovorax benzenivorans*. The type strain has been deposited in the strain collections with the accession numbers DSM 115238^T and NCAIM B.02679^T.

4. CONCLUSIONS AND PROPOSALS

Oil pollution has increased significantly due to globalisation and industrialisation, resulting in oil being one of the most common pollutants today. Of all the BTEX compounds, benzene is the most harmful for human health and the environment (ATSDR 2015, Arnold et al. 2013, Dési 2001). Microorganisms play a key role in ecosystem regeneration, as over time the microbial community adapts to the new carbon source and begins to degrade the pollutant through enzyme-catalysed processes. The removal of the full spectrum of mixed contaminants can be achieved through the cooperation of a community with a diverse enzyme pool (Goldschneider et al. 2006), and cometabolic and other substrate interactions can play a significant role in the degradation process (Littlejohns and Daugulis 2008). The key enzymes in the degradation of aromatic compounds are usually mono- and dioxygenases performing the activation and cleavage of the aromatic ring, and finally, after further conversion steps, the reaction products are utilized through central metabolic pathways (Pérez-Pantoja et al. 2010). The I.2 .C-type catechol-2,3-dioxygenase enzymes play an important role in the degradation of aromatic hydrocarbons in oxygen-limited subsurface environments, as they remain active in the presence of small amounts of oxygen and thus the diversity of these genes in the bacterial community is significant in oxygen-limited environments contaminated with aromatic compounds (Eltis and Bolin 1996, Kukor and Olsen 1996, Táncsics et al. 2012, 2013). The diversity of genes is increased by their spread within the community via horizontal gene transfer (Villa et al. 2019). Today, a relevant scientific objective is to investigate benzene-degrading bacterial communities and strains showing biodegradation activity in subsurface oxygen-limited environments.

Our results show that the question of which members of the bacterial community become dominant depends largely on the type of carbon source and on whether the aromatic hydrocarbon is present in the bacterial environment alone or in a mixture of other BTEX compounds. In addition, the impact of substrate interactions between aromatic compounds on biodegradation should not be overlooked when remediating a contaminated site, as most of the pollutions in natural ecosystems are found in a mixture. Our experiments clearly confirmed that, in addition to the type of aromatic hydrocarbons, the amount of dissolved oxygen has a strong influence on the composition of the aerobic biodegrading bacterial community, as other microorganisms were involved in the biodegradation of benzene under aerobic and microaerobic conditions. Furthermore, not only the enrichted dominant community members can be used to infer the biodegradation capacity of the different genera, but also the results of stable isotope investigations can confirm their prominent role in benzene degradation.

The difference in community composition between the enrichment culture and microcosms may be explained by the fact that the different incubation times may have had a significant effect on the development of the bacterial community. The bacterial community was not only shaped by the fact that a benzene containing medium was provided for five weeks during the incubation period, but also by the fact that the indigenous community was exposed to microaerobic conditions for five weeks and during this time it became a stable microaerobic benzene-degrading community. Indeed, during a five-week enrichment process, competition for available carbon sources may allow those genera that reproduce more rapidly to gain more space, whereas during a community monitoring after a one-week incubation period, it is more likely to detect species that could be marginalised after five weeks of incubation. In addition, genera occupying similar ecological niches, such as *Rhodoferax* and *Azovibrio*, it may occur that species from one or the other genus become dominant.

The aromatic hydrocarbon contaminated subsurface environments provide a great opportunity to isolate new species unknown to science, as the high amount of oil pollution, which are toxic to many organisms, exert strong selection pressure on the endemic community, therefore the bacterial species present in these areas have high genetic diversity, and these type of sites are essentially evolutionary hotspots. However, investigations with our new species also show that classical microbiological methods are still very well applicable to confirm hypotheses established by molecular biological methods and to complement the results, as cultivation techniques can reveal many properties of bacteria that are very important for their usefulness, such as their metabolic capacity.

During the degradation experiments with each bacterial strain, great focus was placed on the ability of the new species to metabolise a BTEX compound as a sole carbon and energy source, and in the case of *Ideonella benzeivorans* B7^{T} and *Acidovorax benzenivorans* D2M1^{T}, to utilise benzene in particular, because in the natural environment, as in a potential inoculant consortium, bacteria that are able to complement each other's degradation spectrums work well together to degrade the petroleum hydrocarbon contaminants. Therefore, it is important that a persistent compound that poses a high environmental risk, such as benzene, is itself a usable carbon source for the studied bacteria. It is rare that a strain with the genetic background necessary for the degradation of aromatic compounds can only degrade benzene and not utilise other monoaromatic hydrocarbons at all, but if not by itself, substrate interactions and cometabolism between BTEX compounds can also allow the degradation of other components by benzene-degrader strains, so that they can be competitive hydrocarbon degraders in the environment in the presence of mixed BTEX contamination as well. However, it is important to note that a dominant community member, such as *Acidovorax benzenivorans* D2M1^{T}, which has a proven ability to metabolise

benzene when released into the environment, is presumably more adaptable than a less competitive species.

Thanks to the results of all our studies, we now have a more accurate picture of the microbial communities of sites contaminated with aromatic hydrocarbons and the role of community-forming microorganisms. Molecular methods have revealed the dominant community components involved in benzene degradation, while stable isotope studies have allowed us to clarify the ecological role of individual bacterial community members, as well as the genome analysis of new species. In particular, we know which taxa can be expected to occur in benzene-contaminated environments under microaerobic conditions (e.g. *Rhodoferax*, *Acidovorax*, *Pseudomonas*, *Malikia*, *Azovibrio*), which knowledge adds a small but important detail to the development of remediation technologies of petroleum-contaminated sites. Classical microbiology has also helped us to identify many of the properties and metabolic capabilities of the new species. The biodegradation potential of the isolated strains has been verified by degradation experiments and genome analysis, and these strains could form the basis of a potential patent for the bioaugmentation of an aromatic hydrocarbon contaminated site, either individually or in consortium.

5. NEW SCIENTIFIC RESULTS

THESIS I.: The investigation of weekly transferred 5th-week microaerobic enrichment cultures containing only benzene or a mixture of toluene, ethylbenzene and xylenes in addition to a predominance of benzene, showed that bacteria of the genus *Pseudomonas* became abundant community members in the presence of mixed aromatic hydrocarbons. In contrast, microorganisms of the genus *Rhodoferax* were more dominant in those media containing benzene only.

THESIS II.: By molecular experiments with stable isotopically labelled benzene-containing microcosms, it was proved that the genera *Pseudomonas* and *Rhizobium* play a role in aerobic conditions and the genera *Malikia* and *Azovibrio* in microaerobic conditions as major competitive benzene degraders in bacterial communities. In the case of *Azovibrio*, the ecological role of this genus in aromatic hydrocarbon degradation was clearly confirmed.

THESIS III.: A bacterial strain belonging to the genus *Pinisolibacter* was successfully isolated from microaerobic benzene-degrading enrichment cultures and was found to possess I.2.B-type catechol-2,3-dioxygenase enzyme based on whole genome analysis. The investigations necessary to describe the species were carried out in accordance with international requirements. Gas chromatography–mass spectrometry (GC-MS) was used to confirm that the strain is capable of complete degradation of ethylbenzene under aerobic conditions. The new species was named *Pinisolibacter aquiterrae*.

THESIS IV.: A bacterial strain belonging to the genus *Ideonella* was successfully isolated from microaerobic benzene-degrading enrichment cultures and described according to international requirements. The strain possesses a phenol degradation gene cluster encoding the I.2.C-type catechol-2,3-dioxygenase gene as part of a complete meta-cleavage pathway. Gas chromatography-mass spectrometry (GC-MS) experiments confirmed that the strain is able to degrade benzene, ethylbenzene and toluene under aerobic and microaerobic conditions. The new species was named *Ideonella benzenivorans*.

THESIS V.: Based on molecular genetic studies, it was confirmed that one of the dominant members of the bacterial communities in benzene-degrading enrichment cultures, belonging to the genus *Acidovorax*, is a new unknown bacterial species possessing the I.2.C-type catechol-2,3-dioxygenase gene. Gas chromatography-mass spectrometry (GC-MS) experiments demonstrated that the strain is able to degrade benzene and ethylbenzene under aerobic and microaerobic conditions. The strain was described according to international requirements and the new species was named *Acidovorax benzenivorans*.

6. PUBLICATIONS RELATED TO THE TOPIC OF THE THESIS

Peer-reviewed, full-text scientific publication in a scientific journal:

Bedics, **A.**, Táncsics, A., Tóth, E., Banerjee, S., Harkai, P., Kovács, B., Kriszt, B. (2022). Microaerobic enrichment of benzene-degrading bacteria and description of *Ideonella benzenivorans* sp. nov., capable of degrading benzene, toluene and ethylbenzene under microaerobic conditions. *Antonie van Leeuwenhoek*, *115*(9), 1113-1128.

Bedics, A., Banerjee, S., Bóka, K., Tóth, E., Benedek, T., Kriszt, B., & Táncsics, A. (2022). *Pinisolibacter aquiterrae* sp. nov., a novel aromatic hydrocarbon-degrading bacterium isolated from benzene-, and xylene-degrading enrichment cultures, and emended description of the genus *Pinisolibacter*. *International Journal of Systematic and Evolutionary Microbiology*, *72*:005229.

Bedics, A., Táncsics, A., Banerjee, S., Tóth, E., Harkai, P., Gottschall, G.G., Bóka, K., Kriszt, B. (2024). *Acidovorax benzenivorans* sp. nov., a novel aromatic hydrocarbon-degrading bacterium isolated from a xylene-degrading enrichment culture. *International Journal of Systematic and Evolutionary Microbiology*, *74*(1), 006219.

Banerjee, S., **Bedics, A**., Tóth, E., Kriszt, B., Soares, A. R., Bóka, K., & Táncsics, A. (2022). Isolation of *Pseudomonas aromaticivorans* sp. nov from a hydrocarbon-contaminated groundwater capable of degrading benzene-, toluene-, m-and p-xylene under microaerobic conditions. *Frontiers in Microbiology*, 13, 929128.

Banerjee, S., **Bedics, A**., Harkai, P., Kriszt, B., Alpula, N., & Táncsics, A. (2022). Evaluating the aerobic xylene-degrading potential of the intrinsic microbial community of a legacy BTEX-contaminated aquifer by enrichment culturing coupled with multi-omics analysis: uncovering the role of *Hydrogenophaga* strains in xylene degradation. *Environmental Science and Pollution Research*, 29 (19), 28431-28445.

Táncsics, A., Banerjee, S., Soares, A., **Bedics, A**., & Kriszt, B. (2023). Combined omics approach reveals key differences between aerobic and microaerobic xylene-degrading enrichment bacterial communities: *Rhodoferax* - a hitherto unknown player emerges from the microbial dark matter. *Environmental Science & Technology*, 57 (7), 2846-2855.

Táncsics, A., **Bedics, A.,** Banerjee, S., Soares, A., Baka, E., Probst, A. J., & Kriszt, B. (2024). Stable-isotope probing combined with amplicon sequencing and metagenomics identifies key bacterial benzene degraders under microaerobic conditions. *Biologia Futura*, *75*(3), 301-311.

7. BIBLIOGRAPHY

Agency for Toxic Substances and Disease Registry (ATSDR). 2015. Benzene toxzine. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service. https://www.atsdr.cdc.gov/sites/toxzine/benzene_toxzine.htmL

Arnold, S. M., Angerer, J., Boogaard, P. J., Hughes, M. F., O'Lone, R. B., Robison, S. H., & Robert Schnatter, A. (2013). The use of biomonitoring data in exposure and human health risk assessment: benzene case study. *Critical reviews in toxicology*, 43 (2), 119-153.

Banerjee, S., Bedics, A., Tóth, E., Kriszt, B., Soares, A. R., Bóka, K., & Táncsics, A. (2022). Isolation of *Pseudomonas aromaticivorans* sp. nov from a hydrocarbon-contaminated groundwater capable of degrading benzene-, toluene-, m-and p-xylene under microaerobic conditions. *Frontiers in Microbiology*, 13, 929128.

Banerjee, S., Bedics, A., Harkai, P., Kriszt, B., Alpula, N., & Táncsics, A. (2022). Evaluating the aerobic xylene-degrading potential of the intrinsic microbial community of a legacy BTEX-contaminated aquifer by enrichment culturing coupled with multi-omics analysis: uncovering the role of Hydrogenophaga strains in xylene degradation. *Environmental Science and Pollution Research*, 29 (19), 28431-28445.

Brown, L. M., Gunasekera, T. S., & Ruiz, O. N. (2017). Draft genome sequence of *Pseudomonas stutzeri* Strain 19, an isolate capable of efficient degradation of aromatic hydrocarbons. *Genome Announcements*, 5, 10-1128.

Chang, M. K., Voice, T. C., & Criddle, C. S. (1993). Kinetics of competitive inhibition and cometabolism in the biodegradation of benzene, toluene, and p-xylene by two *Pseudomonas* isolates. *Biotechnology and Bioengineering*, 41 (11), 1057-1065

Dahal, R. H., Chaudhary, D. K., & Kim, J. (2018b). *Pinisolibacter ravus* gen. nov., sp. nov., isolated from pine forest soil and allocation of the genera *Ancalomicrobium* and *Pinisolibacter* to the family Ancalomicrobiaceae fam. nov., and emendation of the genus *Ancalomicrobium* Staley 1968. *International Journal of Systematic and Evolutionary Microbiology*, 68 (6), 1955-1962.

Dési, I. (Szerk.) (2001): Népegészségtan, Semmelweis Kiadó, Budapest, 273-284., 323-367., 399-402., 421- 450.

Díaz, E. (2004). Bacterial degradation of aromatic pollutants: a paradigm of metabolic versatility. *International Microbiology*, 7:173–180.

Dou, J., Liu, X., & Hu, Z. (2008b). Substrate interactions during anaerobic biodegradation of BTEX by the mixed cultures under nitrate reducing conditions. *Journal of Hazardous Materials*, 158 (2-3), 264-272.

Eltis, L. D., & Bolin, J. T. (1996). Evolutionary relationships among extradiol dioxygenases. *Journal of Bacteriology*, 178 (20), 5930-5937.

Evans, P. J., Ling, W., Goldschmidt, B., Ritter, E. R., & Young, L. Y. (1992). Metabolites formed during anaerobic transformation of toluene and o-xylene and their proposed relationship to the initial steps of toluene mineralization. *Applied and Environmental Microbiology*, 58 (2), 496-501.

Fahy, A., McGenity, T. J., Timmis, K. N., Ball, A. S, (2006): Heterogeneous aerobic benzene-degrading communities in oxygen-depleted groundwaters, *FEMS Microbiology Ecology* 58, 260–270.

Goldschneider, N., Hunkeler, D., & Rossi, P. (2006). Review: Microbial biocenoses in pristine aquifers and an assessment of investigative methods. *Hydrogeology Journal.* 14, 926-941.

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., & Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and Environmental Microbiology*, 79 (17), 5112-5120.

Kukor, J. J., & Olsen, R. H. (1996). Catechol 2, 3-dioxygenases functional in oxygen-limited (hypoxic) environments. *Applied and Environmental Microbiology*, 62 (5), 1728-1740.

Littlejohns, J. V., & Daugulis, A. J. (2008). Kinetics and interactions of BTEX compounds during degradation by a bacterial consortium. *Process Biochemistry*, 43 (10), 1068-1076.

Lee, K., & Gibson, D. T. (1996). Toluene and ethylbenzene oxidation by purified naphthalene dioxygenase from Pseudomonas sp. strain NCIB 9816-4. *Applied and Environmental Microbiology*, 62 (9), 3101-3106.

Lee, I., Ouk Kim, Y., Park, S. C., & Chun, J. (2016). OrthoANI: an improved algorithm and software for calculating average nucleotide identity. *International Journal of Systematic and Evolutionary Microbiology*, 66 (2), 1100-1103.

Lee, Y., Lee, Y., & Jeon, C. O. (2019). Biodegradation of naphthalene, BTEX, and aliphatic hydrocarbons by *Paraburkholderia aromaticivorans* BN5 isolated from petroleum-contaminated soil. *Scientific reports*, 9 (1), 860.

Li, X., Yang, Z., Wang, Z., Li, W., Zhang, G., & Yan, H. (2022). Comparative genomics of *Pseudomonas stutzeri* complex: Taxonomic assignments and genetic diversity. *Frontiers in Microbiology*, 12, 755874.

Lueders, T., Manefield, M., & Friedrich, M. W. (2004). Enhanced sensitivity of DNA-and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environmental Microbiology*, 6 (1), 73-78.

Meier-Kolthoff, J. P., Auch, A. F., Klenk, H. P., & Göker, M. (2013). Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics*, 14, 1-14.

Oh, Y. S., Shareefdeen, Z., Baltzis, B. C., & Bartha, R. (1994). Interactions between benzene, toluene, and p-xylene (BTX) during their biodegradation. *Biotechnology and Bioengineering*, 44 (4), 533-538.

Pérez-Pantoja D, González B, Pieper DH (2010) Aerobic degradation of aromatic hydrocarbons. In: Timmis, K. N. (Szerk.). *Handbook of Hydrocarbon and Lipid Microbiology*. Berlin, Germany: Springer-Verlag Berlin Heidelberg. p. 799–837.

Rodriguez-R, L. M., Castro, J. C., Kyrpides, N. C., Cole, J. R., Tiedje, J. M., & Konstantinidis, K. T. (2018b). How much do rRNA gene surveys underestimate extant bacterial diversity?. *Applied and Environmental Microbiology*, 8 (6), 14-18.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van Horn, D. J., Weber, C. F. (2009): Introducing mothur: open-source platform-independent community-supported software for describing and comparing microbial communities. *Appled and Environmental Microbiology,* 75 (23), 7537-7541.

Táncsics, A., Szoboszlay, S., Szabó, I., Farkas, M., Kovács, B., Kukolya, J., Mayer, Z., Kriszt, B, (2012): Quantification of subfamily I.2.C catechol 2,3-dioxygenase mRNA transcripts in groundwater samples of an oxygen-limited BTEX-contaminated site. *Environmental Science Technology*, 46 (1), 232-240.

Táncsics, A., Farkas, M., Szoboszlay, S., Szabó, I., Kukolya, J., Vajna, B., Kovács, B., Benedek, T., Kriszt, B, (2013): One-year monitoring of meta-cleavage dioxygenase gene expression and microbial community dynamics reveals the relevance of subfamily I.2.C extradiol dioxygenases in hypoxic, BTEX-contaminated groundwater. *Systematic and Applied Microbiology*, 36 (5), 339-350.

Táncsics, A., Banerjee, S., Soares, A., Bedics, A., & Kriszt, B. (2023). Combined omics approach reveals key differences between aerobic and microaerobic xylene-degrading enrichment bacterial communities: Rhodoferax - a hitherto unknown player emerges from the microbial dark matter. *Environmental Science & Technology*, 57 (7), 2846-2855.

Táncsics, A., Bedics, A., Banerjee, S., Soares, A., Baka, E., Probst, A. J., & Kriszt, B. (2024). Stable-isotope probing combined with amplicon sequencing and metagenomics identifies key bacterial benzene degraders under microaerobic conditions. *Biologia Futura*, 75(3), 301-311.

Tindall, B. J., Rosselló-Móra, R., Busse, H. J., Ludwig, W., Kämpfer, P. (2010): Notes on the characterization of prokaryote strains for taxonomic purposes. *Intenational Journal of Systematic and Evolutionary Microbiology,* 60 (1), 249-266.

Tsao, C.-W., Song, H.-G., & Bartha, R. (1998). Metabolism of benzene, toluene, and xylene hydrocarbons in soil. *Applied and Environmental Microbiology* 64*, 4924–9*.

Vallenet, D., Labarre, L., Rouy, Z., Barbe, V., Bocs, S., Cruveiller, S., Lajus, A., Pascal, G., Scarpelli, C., & Medigue, C. (2006). MaGe: a microbial genome annotation system supported by synteny results. *Nucleic Acids Research*, 34 (1), 53-65.

Vallenet, D., Engelen, S., Mornico, D., Cruveiller, S., Fleury, L., Lajus, A., Rouy, Z., Rosche, D., Salvignol, G., Scarpelli, C., & Médigue, C. (2009). MicroScope: a platform for microbial genome annotation and comparative genomics. *Database*, *2009*.

Villa, T. G., Feijoo-Siota, L., Sánchez-Pérez, A., Rama, J. R., & Sieiro, C. (2019). Horizontal gene transfer in bacteria, an overview of the mechanisms involved. *Horizontal gene transfer: breaking borders between living kingdoms*, 3-76.

Winderl, C., Penning, H., Netzer, F. V., Meckenstock, R. U., & Lueders, T. (2010). DNA-SIP identifies sulfate-reducing Clostridia as important toluene degraders in tar-oil-contaminated aquifer sediment. *The ISME journal*, 4 (10), 1314-1325.

Internet references:

http1: http://microbial-genomes.org6

http2: https://ggdc.dsmz.de/

http3: http://www.mothur.org/wiki/MiSeq_SOP