Microbial biodegradation of aromatic hydrocarbons in subsurface environments: elucidating the genetic background of microaerobic degradation by stable isotope and „omics" approaches

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Final Research Report

by

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Introduction: background and aims of the study

The monoaromatic hydrocarbons, such as the carcinogenic benzene, and others like toluene, ethylbenzene and xylenes (BTEX-compounds) are among the most frequent groundwater contaminants in Hungary. Due to their relatively high water solubility, a soil contamination can considerably threaten subsurface aquifers, the main drinking water resources. Therefore, the cleanup of the polluted ecosystems is always obligatory by law in Hungary. Although several methods are available to eliminate subsurface hydrocarbon contaminations, bioremediation is the only relevant and environmentally sound method to treat large scale BTEX contaminations. Since microbial degradation of these monoaromatic hydrocarbons readily occurs under aerobic conditions, oxygen supply of the contaminated ecosystem is a frequently applied method to enhance aerobic degradation of the contaminants. This is necessary because hydrocarbons stimulate the metabolic activity of indigenous aerobic microbial populations, resulting in a rapid depletion of dissolved oxygen in subsurface environments. Unfortunately, little is known about how different aromatic hydrocarbon-degrading microbial communities evolve under different levels of dissolved oxygen availability. It is known that trace amounts of oxygen can support aerobic degradation of benzene (Yerushalmi et al., 2002; Aburto et al., 2009) and that certain functional genes (catechol dioxygenases) playing key role in the degradation of aromatic compounds are transcribed even under microaerobic conditions (Táncsics et al., 2012). Therefore, it is conceivable that much smaller level of aeration of the contaminated subsurface environment would be sufficient to enhance aerobic degradation of the contaminants. In the light of environmental sustainability, it would be beneficial to uncover the diversity of microbes capable of degrading benzene or xylenes under microaerobic conditions and to reveal the genetic background of microaerobic BTEX-degradation. Accordingly, major aims of the project were the following:

- To reveal the diversity of those microbes and ring-cleavage (C23O) enzymes, which play key role in the microarobic degradation of benzene by using enrichment and stable isotope probing-based approaches.
- To reveal the diversity of bacteria capable of microaerobic xylene degradation.
- To isolate bacterial strains capable of degrading benzene or xylene under microaerobic conditions for the purpose of new species descriptions and to study the genetic background of the degradation.

Results of the project

Enrichment I: Investigating bacterial communities evolved under microaerobic conditions in the presence of benzene *versus* all of the six BTEX-compounds

Aims and design of the experiment

The aim of enrichment I was to test whether the BTEX-contaminated groundwater sediment of the Siklos site is appropriate to use it as inoculum during the research project. This was necessary since previously we observed in case of another contaminated site, that the success of microaerobic enrichment of benzene-degrading bacterial community is not obvious (Révész et al, 2020). In other words, this was a pilot experiment. Two types of triplicate microaerobic enrichments were established: three of the enrichments contained only benzene as sole source of carbon (1 mM), while another three enrichments contained benzene (B) as the major carbon source, and toluene, ethylbenzene and xylene (TEX) were present as minor components (B:TEX ratio 5:1). The dissolved oxygen concentration in the enrichments was measured non-invasively by a fiber optic sensor and kept at 0.5 mg/L. The concentration of the aromatic hydrocarbons was measured by GC-MS. The enrichments were transferred weekly for for 5 weeks, after which the evolved bacterial communities were investigated by 16S rRNA gene amplicon sequencing and bacterial strain isolation on R2A agar plates.

Results

In the benzene-degrading microaerobic enrichment the major community players were members of the genera *Rhodoferax* and/or *Acidovorax* based on the results of the 16S rRNA gene amplicon sequencing results. In two of the enrichments *Rhodoferax* was overwhelmingly dominant by showing 58-63% relative abundance, while in the third enrichment *Acidovorax* was the dominant (79%). Interestingly, members of the genus *Pseudomonas* showed relatively low abunce in these enrichments (3-16%). In contrast, in the microaerobic enrichments containing all of the BTEX-compounds, members of the genus *Pseudomonas* were the most dominant (49-89%), followed by *Acidovorax* (7-40%). Besides, in one of the triplicates *Rhodoferax* was detected with 10% abundance.

Altogether 26 bacterial strains were isolated from the enrichments (13 from the benzene and another 13 from the BTEX-degrading). In case of the benzene-degrading enrichments the

isolates belonged to the genera *Pseudomonas* (4), *Rhizobium* (2), *Rhodococcus* (2), *Brucella* (2), *Xanthobacter* (1), *Ideonella* (1) and *Pinisolibacter* (1). In case of the BTEX-degrading enrichment the isolates belonged to the genera *Pseudomonas* (6), *Rhizobium* (2), *Brucella* (2), *Brevundimonas* (2) and *Ferrovibrio* (1).

Among strains isolated from the microaerobic benzene-degrading enrichments, only one isolate, *Ideonella* sp. strain B7 harboured subfamily I.2.C-type C23O gene, suggesting that this bacterium may have the ability to degrade benzene under microaerobic conditions. Moreover, its closest relative was *Ideonella dechloratans* CCUG 30977^{T} with 98.4% 16S rRNA gene similarity, assuming that strain B7 represents a novel species of the genus *Ideonella*. Due to this, we performed a polyphasic approach to describe this new species. Whole-genome analysis of strain B7 revealed that this bacterium harboured a gene cluster, which encoded a multicomponent phenol-hydroxylase, the I.2.C-type C23O gene, and a complete lower meta-pathway for the degradation of monoaromatic hydrocarbons. As predicted by the genome analysis, it was observed that strain B7 was capable of degrading both aerobically and microaerobically benzene, toluene and ethylbenzene as sole source of carbon and energy.

Among the strains another new species candidate was found, strain B13, which was most closely related to *Pinisolibacter ravus* E9^{T} by showing 97.36% 16S rRNA gene similarity. Due to this low similarity, we decided to perform the analyses required for the species description. Moreover, the whole-genome analysis revealed that strain B13 has the genetic potential to degrade monoaromatic hydrocarbons. A catechol 2,3-dioxygenase (subfamily I.2.B-type C23O) gene was found in a gene cluster, encoding a partial *meta*-cleavage pathway. The closest relative of this C23O gene was found in a *Methylocella tundrae* isolate, although with a considerably low sequence similarity (80%). The *meta*-cleavage gene cluster containing the C23O gene was found to be partial, since it lacks the genes encoding for the upper pathway enzymes. On the other hand, a gene cluster encoding a toluene-monooxygenase was found in the genome, which could complement the partial *meta*-cleavage gene cluster, providing aromatic hydrocarbon-degrading ability to strain B13^{T}. Eventually, **strain B13 was validly described as *Pinisolibacter aquiterrae* B13^{T} by Bedics et al. (2022) in International Journal of Systematic and Evolutionary Microbiology (Q1, IF: 2.8).**

Results of enrichment I experiment together with the valid description of *Ideonella benzenivorans* B7^{T} was published by Bedics et al. (2022) in Antonie van Leeuwenhoek (Q2, IF: 2.6).

Enrichment II: Revealing the diversity of bacteria capable of degrading benzene under microaerobic conditions by a stable isotope-probing (SIP) approach

Aim and design of the experiment

The aim of the second enrichment experiment was to clearly identify those bacterial taxa, which play major role in the microaerobic degradation of benzene. Accordingly, sediment sample was taken from the Siklós BTEX-contaminated site and used to establish triplicate aerobic and microaerobic enrichments. The sole carbon source in the enrichments was isotopically labelled benzene ($^{13}C_6$) in 0.5 mM final concentration. The dissolved oxygen concentration in the microaerobic enrichments was kept at 0.5 mg/L, while it was kept between 6-8 mg/L in the aerobic enrichments. The concentration of benzene in the enrichments was monitored by GC-MS. After complete degradation of benzene, DNA was isolated from the enrichment microbial communities. Following DNA isolation, 16S rRNA genes were amplified from the non-density resolved DNA samples and bacterial diversity of the enrichment communities were revealed by Illumina 16S rRNA gene amplicon sequencing. In the next step DNA samples were submitted to isopycnic centrifugation, performed in cesium-chloride (CsCl density-gradient centrifugation), thus heavy and light DNA fractions were separated. The heavy DNA fraction contained mostly the DNA of those bacteria, which were able to use the benzene as sole source of carbon, since their DNA contained the ^{13}C originated from the $^{13}C_6$-benzene. Accordingly, 16S rRNA genes were amplified from the DNA fractions and were compared by Illumina amplicon sequencing. As a result, we were able to clearly identify bacterial taxa performing benzene-degradation under microaerobic conditions.

Results

At first, microbial communities were investigated by using the non-density resolved DNA samples obtained from each of the enrichments. In the aerobic enrichments members of the genus *Pseudomonas* were overwhelmingly dominant (with relative abundance values between 40-51%), *Rhizobium* (10-15%), *Thauera* (12-15%). The major *Pseudomonas* OTUs were related to *P. aromaticivorans* (35-45%) and members of the *P. stutzeri* complex (4-5%). Notable minor community members detectable in all of the aerobic enrichments were *Zoogloea*

(2-3%), *Sulfuritalea* (~2%) and *Malikia* (~1.5%). In the case of the microaerobic enrichments, members of genera *Malikia* (26-40%) and *Azovibrio* (20-28%) dominated the communities, which later lineage was completely missing from the aerobic enrichments. The genus *Malikia* was represented by an OTU most closely related to *M. spinosa*. Although the type strain of this bacterium was isolated from a pristine freshwater, it has been shown by us earlier that this bacterium can acquire the ability to degrade aromatic hydrocarbons and can play major role in aerobic benzene degradation (Révész et al. 2020). The *Azovibrio* genus was represented by an OTU most closely related to *A. restrictus*, but only at 95.7% 16S rRNA gene sequence similarity. Nevertheless, the role of *Azovibrio* in the degradation of BTEX has never been reported earlier. It was also observable that the abundance of genus *Pseudomonas* considerably decreased and became to be minor player of the microaerobic communities. This decrease was due to the fact that the abundance of the *P. aromaticivorans* OTU was only 0.5-1% in the microaerobic enrichment communities, while the abundance of the other *Pseudomonas* OTU remained between 4-6%. Overall, non-density resolved DNA samples indicated that highly different benzene-degrading microbial communities evolved under aerobic *versus* microaerobic conditions.

In order to clearly identify the bacterial lineages, which were degrading the isotopically labelled benzene ($^{13}C_6$-benzene), community DNA samples were density resolved by isopycnic gradient ultracentrifugation. In case of the aerobic enrichments, genera *Pseudomonas* and *Rhizobium* were clearly identified as benzene-degraders, due to the notable increase in their abundances in the heavy DNA fraction compared to that of the light fraction. In case of the *P. aromaticivorans* OTU, its 36.8% abundance observed in the light fraction increased to 43% in the heavy DNA fraction. However, in case of the *P. stutzeri* OTU, an opposite trend was observable, as its abundance was higher in the light fraction (4% vs 2.7%). Accordingly, *P. aromaticivorans* was identified as a prominent benzene degrader under clear aerobic conditions. On the other hand, this OTU was hardly detectable in the heavy DNA fraction of the microaerobic enrichments, reaching only 0.15% abundance. As mentioned above, *Rhizobium* was identified as benzene-degrader, since its abundance was considerably higher in the heavy DNA fraction than in the light one (23% *vs* 12.5%). In case of the microaerobic enrichments the two major players, *Malikia* and *Azovibrio* were clearly identified as benzene-degraders. DNA of both lineages showed considerably higher abundance in the heavy fraction than in the light fraction (42% vs 33% and 33% vs 26%, respectively). Overall, based on the results of the SIP-based approach two major taxa were identified as microaerobic degraders: *Malikia* and

Azovibrio. Besides, the results confirmed the observation of the previous enrichment, that members of the genus *Pseudomonas* play marginal role, if any, in the degradation of benzene under microaerobic conditions.

The manuscript summarizing the results of the SIP-approach is under preparation and planned to be submitted to Environmental Pollution (D1, IF: 8.9) during autumn 2023.

Enrichment III: Revealing the aerobic xylene-degrading bacterial diversity of a petroleum hydrocarbon contaminated aquifer

Aim and design of the experiment

The aim of enrichment III was to reveal the aerobic xylene-degrading potential of the microbial communities of the Siklós BTEX-contaminated site. Prior to the microaerobic enrichment experiment (enrichment IV) we wanted to see that the microbial community here does not lack this ability, and samples of the Siklós site will be appropriate to serve as inoculum for the microaerobic enrichment. In other words, this was a pilot experiment. Another aim was to reveal, whether different populations are active in the degradation of the three different xylene isomers (*m*-,*p*- and *o*-xylene). Accordingly, aerobic duplicate enrichments were set up with one of the xylene isomers. The evolved bacterial communities were investigated after five consecutive transfers (with weekly transfers) by 16S rRNA gene Illumina amplicon sequencing and strain isolation on R2A agar plates.

Results

The bacterial community of the *m*-xylene-degrading enrichment was dominated by members of the genera *Sediminibacterium* (27.1%), *Pseudomonas* (22.8%), and *Polaromonas* (18.4%). Whereas in *p*-xylene-degrading enrichment, members of the genus *Pseudomonas* overwhelmingly dominated the community by showing 64% relative abundance. Besides, members of some other genera like *Acidovorax* (13.2%), *Enterobacter* (5.1%), *Sediminibacterium* (4.6%), and *Hydrogenophaga* (3.9%) were detectable with prominent abundance as well. However, the *o*-xylene-degrading enrichment showed an altogether different community structure. Though *Pseudomonas*-related bacteria were present in the community but only with 14% abundance. *Acidovorax* (24.9%) was the most dominant genus

along with *Sulfuritalea* (22.8%), *Rhodococcus* (14.6%), *Chryseobacterium* (8.4%), and *Hydrogenophaga* (4.4%).

Based on different colony morphology and growth pattern total number of 21 strains have been isolated, among which six isolates originated from *m*-xylene-degrading enrichment, eight isolates from *p*-xylene-degrading enrichment, and seven isolates from *o*-xylene-degrading enrichment. Strains isolated from the *m*-xylene-degrading enrichment belonged to the genera of *Acidovorax*, *Pseudacidovorax*, *Polaromonas*, *Lysobacter*, *Pseudomonas*, and *Achromobacter*. Amid those strains, *Acidovorax*, *Pseudacidovorax*, *Polaromonas*, and *Pseudomonas* possessed subfamily I.2.C-type C23O gene, which was sequenced further to study functional gene diversity in the isolated community members. Strains isolated from the *p*-xylene-degrading enrichment were members of the genera *Hydrogenophaga*, *Acidovorax*, *Mycolicibacterium*, *Pseudomonas*, and *Enterobacter*. The screening of subfamily I.2.C-type C23O gene showed that *Hydrogenophaga*, *Acidovorax*, and *Pseudomonas* strains harbored such a gene. Overall, it was be concluded, that the microbial community at the Siklós BTEX-contaminated site of Hungary had the metabolic potential to aerobically degrade all isomers of xylene. Polyphasic analysis of the enrichments revealed that distinctly different bacterial communities played role in the degradation of the different xylene isomers. Still, members of the genera *Pseudomonas* and *Acidovorax* were abundant community members in all of the enrichments, while bacteria belonging to the genera *Rhododoccus* and *Chryseobacterium* were key players only in the *o*-xylene-degrading enrichment cultures.

Results of enrichment III were published by Banerjee et al. (2022) in Environmental Science and Pollution Research (Q1, IF: 5.8).

Due to the fact that members of the genus *Acidovorax* were abundant community members both in enrichment I and enrichment III, we decided to investigate such an isolate deeper. Although *Acidovorax* sp. strain D2M1 was isolated from the *m*-xylene-degrading enrichment of enrichment III experiment, it was able to degrade benzene under microaerobic conditions. The whole genome of the strain was sequenced and we defined the position of the subfamily I.2.C-type C23O in the genome. Besides, it was found that the genome contains a catechol 1,2-dioxygenase gene as well. Phylogenomic analysis was also performed, and the results suggested that strain D2M1 can be delineated at species level from its closest relative *A. delafieldii*, due to the 39% dDDH and 90% OrthoANI values showing with the type strain

DSM64^T. Consequently, we performed the polyphasic analysis process needed to describe strain D2M1 as a new species of the genus *Acidovorax*. **The manuscript, which describes *Acidovorax benzenivorans* sp. nov. was submitted by Bedics et al. (2023) to International Journal of Systematic and Evolutionary Microbiology (Q1, IF: 2.8) at 6th of June 2023, and it is under review at the time of writing.** We also started a transcriptomic analysis to investigate gene expression of strain D2M1 while degrading benzene under microaerobic conditions, but the first attempt failed due to low quality RNA. Thus, this experiment will be repeated.

Enrichment IV: Investigating the difference between aerobic and microaerobic xylene-degrading bacterial communities by a multiomics approach

Aim and design of the experiment

The aim of enrivhment IV was to reveal the possible differences between xylene-degrading bacterial communities enriched under aerobic *versus* microaerobic conditions and to identify bacteria capable of degrading xylene under microaerobic conditions. To achieve this, a polyphasic study was performed in which the enrichment culturing was coupled with a multi-omics approach, including: 16S rRNA gene amplicon sequencing, genome-resolved metagenomics and genomic analysis of selected xylene-degrading isolates. The results of this study have provided new insight into the differences between aerobic and microaerobic xylene-degrading bacterial communities.

Enrichments were performed similarly as in case of the previous enrichments. Briefly, triplicate aerobic (DO 6-8 mg/L) and microaerobic (DO 0.5 mg/L) enrichments were set up and transferred for 5 consecutive weeks, followed by the multiomics analysis and bacterial strain isolation.

Results

In the aerobic enrichments, members of the genus *Pseudomonas* were the most dominant (with relative abundance values between 47-59%), followed by *Sphingobium* (20-30%) and *Acidovorax* (4-6%). In the case of the microaerobic enrichments, members of the

genus *Pseudomonas* were the most dominant (~50% abundance value). Interestingly, *Sphingobium* was not detected in any of the microaerobic enrichments. Instead, members of the genera *Azovibrio* (25% relative abundance in enrichment MIC1) or *Rhodoferax* (20 and 31% relative abundance in enrichments MIC2 and MIC3, respectively) were the second most dominant group in the enriched bacterial communities. Based on these results *Rhodoferax* and the *Azovibrio* lineages were suggested to play role in the microaerobic degradation of benzene together with certain members of the genus *Pseudomonas*.

It is well known that a certain phyletic lineage of the genus *Rhodoferax* is associated with aromatic hydrocarbon-contaminated subsurface environments. It was observed previously at the Siklos site, that both at the center and the fringes of the contaminant plume an uncultivated lineage of the genus *Rhodoferax* was the most dominant community member. Nevertheless, a representative of this lineage has not been cultivated from petroleum hydrocarbon contaminated environment. In order to reveal the genome of the *Rhodoferax*-related bacterium, which was abundant in the microaerobic enrichments, genome resolved metagenomic analysis of sample enrichment MIC3 was performed. Altogether twelve, high quality metagenome-assembled genomes (MAG) were reconstructed from the metagenome dataset of enrichment MIC3. The *Rhodoferax* MAG was 4.70 Mb with a G+C content of 56.45%, but did not contain SSU sequences, although its completeness was >97%. Both the MiGA and the UBCG pipelines indicated that the recovered genome belonged to the genus *Rhodoferax*, showing the closest relationship to *Rhodoferax* sp. strain MIZ03, *R. fermentans* and *R. ferrireducens*. Analysis of the genome revealed the presence of a gene cluster (tmoABCDEF) encoding a toluene-4-monoxygenase. This gene cluster contained a gene which encoded an outer membrane transport protein (TmoX), most probably playing a role in the transport of monoaromatic hydrocarbons from the extracellular environment to the periplasm. Moreover, transcriptional regulator genes tmoS and tmoT were also part of this gene cluster. Around 20 kilobases downstream of the gene cluster coding for Tmo, another cluster was found that coded for a multicomponent phenol hydroxylase (mPH) and a complete *meta*-cleavage pathway (mPH1 cluster). Additionally, another contig coded a second mPH together with *meta*-cleavage enzymes (mPH2 cluster). Interestingly, the extradiol ring-cleavage dioxygenase (EDO) enzymes coded in both mPH clusters were not subfamily I.2.C-type EDO enzymes. Instead, they formed a distinct, and as yet undefined subfamily of EDO enzymes on the phylogenetic tree. According to the evolutionary classification of extradiol dioxygenases this new subfamily was designated as I.2.I. by us.

Besides the molecular approach, standard bacterial strain isolation on R2A was also utilized in order to gain more data on the bacterial communities present in the enrichments and to explain the observed differences in the bacterial community structures. Altogether, 43 strains were isolated from the aerobic and microaerobic enrichments. For further genomic analysis, two strains representing a characteristic group of bacteria from either the aerobic or the microaerobic enrichments were chosen: *Sphingobium* sp. strain AS12 isolated from the aerobic enrichments, and *Pseudomonas* sp. strain MAP12 isolated from the microaerobic enrichments.

Although strain AS12 could be identified as *S. terrigena* based on its 16S rRNA gene sequence, genome relatedness analysis with the type strain *S. terrigena* EO9^T yielded an ANI value of 92% and a dDDH value of 48.8%, hinting at the possibility that strain AS12 represents a yet undescribed lineage of the genus *Sphingobium*. Its genome proved to be 4.86 Mbp with a G+C content of 62.5% (NCBI accession number: JAHRGM000000000.1). Annotation of the genome identified the genetic toolkit needed for the utilization of various aromatic compounds. Genes which are responsible for aromatic hydrocarbon degradation were found on a ~62 Kbp large scaffold of the genome, including a C23O gene, which encoded a subfamily I.2.B-type EDO enzyme. Another important thing to note is that the inability to reduce nitrate is a common characteristic of all *Sphingobium* species. Consequently, they are less competitive aromatic compound degraders in oxygen-limited environments compared to those bacteria (e.g *Rhodoferax*) which may use oxygen and nitrate simultaneously as electron acceptors under microaerobic conditions.

Strain MAP12, isolated from the microaerobic enrichments, showed the closest relationship to *Pseudomonas* linyingensis LYBRD3-7^T, with 98.4% 16S rRNA gene sequence similarity, followed by *Pseudomonas sagittaria* JCM 18195^T (98.2% similarity). Whole-genome analysis of strain MAP12 yielded a 4.39 Mbp large genome with a G+C content of 65.7%. The ANI and dDDH analyses of the closest relatives resulted in OrthoANI values <89% and dDDH values <53%, indicating that MAP12 represents a yet undescribed lineage of the genus *Pseudomonas*, and was described by us as *P. aromaticivorans*. This bacterium was positive for xylE3 PCR, thus it harbored a subfamily I.2.C-type C23O in its genome. Annotation of the genome confirmed this result and revealed that this gene was part of a partial toluene/xylene degradation gene cluster (containing upper TOL pathway genes, but lacking lower *meta*-cleavage genes). Its sequence similarity with the C23O II gene of *Pseudomonas putida* MT15, which is an archetype of subfamily I.2.C-type C23O genes, was ~82%. Results of the xylene degradation experiment using strain MAP12 indicated that this *Pseudomonas*

strain could only use *p*-and *m*-xylene as its sole source of carbon and energy, and was unable to utilize *o*-xylene. It was also observed that there was no difference in the degradation efficiency of *m*-xylene under aerobic and microaerobic conditions by strain MAP12. (In order to investigate the microaerobic degradation of *m*-xylene by strain MAP12, a transcriptomic study was performed. The RNA sequencing was performed in August 2023, thus the analysis of the acquired data is an ongoing process.) Besides the subfamily I.2.C-type C23O, three subfamily I.2.A-type C23O genes and, surprisingly, a subfamily I.2.I-type C23O were identified in the whole-genome sequence.

The description of the new species *Pseudomonas aromaticivorans* was published by Banerjee et al. (2022) in Frontiers in Microbiology (Q1, IF: 5.2).

In conclusion, the enrichment approach in combination with multi-omics analysis yielded significant new knowledge on xylene biodegradation by subsurface microbiota. Our results confirmed that a *Rhodoferax* genus related lineage, which is usually abundant in aromatic hydrocarbon contaminated subsurface environments worldwide, contains prominent degraders and can possess the genomic ability to degrade not just xylene, but benzene and toluene as well by using aerobic degradation pathways. It is important to emphasise that *Rhodoferax* was only abundant under microaerobic conditions, although the EDO enzymes coded by this bacterium were not subfamily I.2.C-type EDOs. Indeed, they belonged to a newly defined EDO subfamily, designated as I.2.I. Overall, we observed remarkable differences in the structure of the bacterial communities between the aerobic and microaerobic xylene-degrading enrichments, driven primarily by (i) the mechanism of aromatic ring-activation (monooxygenation versus dioxygenation), (ii) the type of EDO enzymes and (iii) the ability of the degraders to respire with nitrate.

The results of enrichment IV were published by Táncsics et al. (2023) in Environmental Science & Technology (Nature Index journal, D1, IF: 11.4).

Conclusions

As main results of the research project, we achieved the following major findings:

- Distinctly different microbial communities take part in the degradation of aromatic hydrocarbons under microaerobic or clear aerobic conditions.
- We identified three microbial taxa, which play key role in the degradation of benzene under microaerobic conditions, and these belong to the genera *Rhodoferax*, *Azovibrio* and *Malikia*.
- Members of the genus *Pseudomonas* play marginal role in the degradation of benzene under microaerobic conditions.
- Three microbial taxa were identified as key xylene degraders under microaerobic conditions, and these belong to the genera *Rhodoferax*, *Azovibrio* and *Pseudomonas.*
- Besides subfamily I.2.C-type catechol 2,3-dioxygenases, we identified a new subfamily, I.2.I, which possibly also plays role in the microaerobic degradation of aromatic hydrocarbons.
- Four new, aromatic hydrocarbon-degrading bacterial species we described during the project: *Ideonella benzenivorans*, *Pinisolibacter aquiterrae*, *Pseudomonas aromaticivorans* and *Acidovorax benzenivorans*.

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