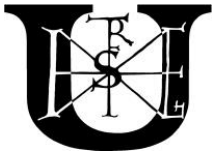

Szent István University

Ph.D. thesis

Degradation of monoaromatic hydrocarbons in a hypoxic BTEX contaminated groundwater

Farkas Milán

Gödöllő

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Ph.D. School

Name: Ph.D. School of Environmental Sciences

Discipline: Environmental Sciences

Leader of School: Csákiné Dr. Erika Michéli,
Ph.D. Professor, Head of department
Szent István University
Faculty of Agricultural and Environmental Sciences
Institute of Environmental Sciences Department of Soil sciences and Agrochemistry

Supervisors: Dr. András Táncsics
Senior Research Fellow,
Regional University Center of Excellence in Environmental Industry

Dr. Sándor Szoboszlay
Associate professor, Head of department
Institute of Aquaculture and Environmental Safety
Department of Environmental Safety and Ecotoxicology

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Approval of School Leader Approval of Supervisors

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1. Introduction and aims

According to some forecast nowadays we have reached the peak in global oil production, probably no more supergiant fields will be discovered at the mainland. However the exploitation and refining of crude oil still decades away, and while oil is existing pollutions could appear. Petroleum hydrocarbons are the most frequent contaminants of soil, groundwater or marine environments. Many of these pollutants can be harmful to human health, we can find carcinogen, teratogen and mutagen compounds among them.

However, most studies have addressed either strictly aerobic or anaerobic degradation of aromatic hydrocarbons, little is known about the hypoxic processes. In subsurface ecosystems, the availability of oxygen is often restricted. Therefore, study of microbial communities adapted to microaerob conditions is always required.

During our work attempt was made to

- monitor the meta-cleavage dioxygenase gene expression and microbial community dynamics of a BTEX contaminated site
- identify the microaerob toluene degraders by stable isotope probing
- enrich and isolate specific groups of microorganisms with capability of degrading monoaromatic hydrocarbons under hypoxic conditions.

2. Materials and methods

To achieve our aims three different investigations, experimentations were carried out. In the first study, meta-cleavage dioxygenase gene expression and microbial community dynamics were monitored monthly for a year in a well-known hypoxic, BTEX-contaminated shallow groundwater. In the second study we identify the microaerob toluene degraders by stable isotope probing. Finally an attempt was made to enrich the facultative anaerob degraders from the microbial community.

2.1 Long term monitoring of microbial community in hypoxic, BTEX-contaminated groundwater.

For the long term monitoring a Hungarian BTEX contaminated hypoxic site where chosen witch was contaminated by leaking oil storage tanks. Groundwater samples were taken from the center of the contaminant plume (ST2 well) monthly between May 2010 and May 2011. Key physical and chemical parameters, such as dissolved oxygen (DO) concentration, redox potential (EH), temperature and pH were measured on-site by means of portable field sensors (HANNA Instruments®, USA). Concentrations of the BTEX compounds and physicochemical parameters were determined by an accredited analytical laboratory (Wessling Hungary Ltd.).

In order to cover a wide range of meta-cleavage dioxygenases four main gene groups were targeted by PCR. Amplification of C23O genes encoding subfamily I.2.A (*Pseudomonas*) and I.2.B (mainly *Sphingomonas*) extradiol dioxygenases were essayed by using the group-specific primer sets XYLE1 and XYLE2 designed by Hendrickx et al. (2006). For the group-specific detection of C23O genes encoding subfamily I.2.C extradiol dioxygenases, a new degenerate primer set was designed and designated as “XYLE3”. Since ethyl-benzene was a

major contaminant at the investigated site, expression of 3-isopropylcatechol (3-IPC) 2,3-dioxygenase genes was also investigated. Amplification of these target sequences was carried out by using the primer set EXDO-K2 according to Brennerova et al. (2009).

Amplification of 16S rRNA genes for cloning purposes was carried out by using the bacterial primers 27F (Lane 1991) and 519R (Turner et al. 1999), while for T-RFLP purposes the forward primer was fluorescently labeled (VIC). To obtain molecular fingerprints after amplification, 16S rDNA amplicons were digested with the restriction enzyme *Rsa*I (GT↓AC) (Thermo Scientific). For the 16S rDNA clone libraries, more than 100 clones were sequenced for each transformation, while 48 clones were sequenced for the catechol dioxygenase clone libraries. Clone libraries revealed the presence of six different clusters of subfamily I.2.C C23O genes. In order to reveal expression pattern changes that may occurred during the monitoring period we developed a SNuPE assay. This quasi fingerprinting of gene expression provided opportunity to link the investigated function to specific microbial populations. Relationships between 16S T-RFLP and SNuPE fingerprinting results were analyzed with principal component analysis (PCA) ordination combined with vector fitting.

2.2 The stable isotope probing experiment

Sediment rich samples were taken from the bottom of a monitoring well (ST-2) in 2015 April. Triplicates of 5g homogenously mixed sediment material were transferred into 15 pieces of sterile 100-mL serum bottle containing 50 mL of artificial groundwater medium (Winderl et al. 2010). 5 μm cAMP, vitamin and mineral solution were added to the medium (Bruns et al. 2002). Dissolved oxygen concentration in the bottles was set to 0,5 mg/L, and kept between 0,5 and 0 mg/L throughout the experiment. 5 μL of either non-labeled (^{12}C) or fully labeled ($^{13}C_7$) toluene (Sigma-Aldrich) were injected to six-six microcosms.

Three abiotic control bottles (autoclaved three times) amended with unlabeled toluene were also prepared to exclude abiotic toluene loss or redox reactions. As it was expected rapid depletion of toluene was observed.

To minimize the cross-feeding interactions sediments were collected from microcosms after 3 and 7 day of incubation. DNA extract was loaded onto a gradient medium of CsCl and centrifuged (180 000 *g*, ~68 h). Twelve fractions from each gradient were collected from 'heavy' to 'light'. After qualitative and quantitative analysis eight DNA fractions of each gradient were selected for bacterial 16S rRNA T-RFLP fingerprinting. FAM labeled amplicons were generated with the primers Ba27F and 907R (Muyzer and Smalla 1998) and restricted using *Rsa*I enzyme. Non-density-resolved total DNA extracts from the inoculum and the selected gradient fractions were subjected to 16S rDNA amplicon pyrosequencing.

C23O amplicons generated with the primer set XYLE3F/XYLE3R were cloned and sequenced from the initial sediment sample, as well as from selected "heavy" and "light" DNA fractions of the day 3 ^{13}C-toluene SIP gradient.

2.3 Conditions of Fe(III)-reducing enrichment

Continuous monitoring of the microbial community structure in the center of the contaminant plume indicated the varying dominance of a *Rhodoferax* phylotype, which can be linked to aromatic hydrocarbon degradation. Moreover, emergence of a *Geobacter* population was observed in the center of the plume from year 2013. Based on all abovementioned data, the main objective of the third study was to enrich and identify these Fe(III)-reducing microbes and understand they role in the degradation of BTEX compounds.

Groundwater sample from the Siklós BTEX contaminated site was incubated anaerobically for two weeks to enhance the nitrate depletion. According to the guidelines for enrichment of purple non-sulfur bacteria (especially *Rhodoferax*

genus) four different phosphate buffer based acetate enrichments were set up: (i) supplemented with 0.05% (w/v) yeast extract and NH4Cl; (ii) supplemented solely with 0.05% (w/v) yeast extract; (iii) supplemented with NH4Cl as sole fix nitrogen form; and finally (iv) omitting both yeast extract and NH_4Cl. Minimal enrichment media were inoculated with groundwater samples under anaerobic circumstances. Fe(III)NTA was added to the media to provide Fe(III) as sole electron acceptor.

After five constitutive transfers, DNA was isolated from the microcosms. The methods of sequence-aided 16s rRNA T-RFLP with restriction enzyme *FspB*I (C↓TAG) and clone libraries were used to follow changes in the community structure during the enrichments.

Total environmental DNA isolated from the initial groundwater sample was used for high-throughput sequencing using an Ion Torrent PGM instrument (Life Technologies). To find correlations between microbial community and enrichment media composition canonical correspondent analysis was done.

Detection of subfamily I.2.C-type catechol 2,3-dioxygenases (C23O) was essayed by using primers XYLE3F and XYLE3R (Táncsics et al. 2013). Amplification of the bssA - benzylsuccinate synthase alpha subunit fragments was also attempted by using the primer set 7772F and 8546R of Winderl et al. (2007).

2.4 One year monitoring of a microbial community in a hypoxic BTEX contaminated groundwater

The groundwater community in the BTEX contaminated ST2 well was dominated by Betaproteobacteria, mainly by members of the genera *Rhodoferax* and *Azoarcus*, and unknown bacteria related to Rhodocyclaceae.

Although the community composition was quite stable, remarkable shifts in the dominancy of these major taxa were observed. These shifts most probably occurred due to the fact that the investigated groundwater was shallow, thus hydrologically dynamic.

In accordance with our results, numerous previous studies have reported on the prevalence of Betaproteobacteria in oxygen-limited, aromatic hydrocarbon-contaminated groundwaters (Fahy et al. 2006, Martin et al. 2012, Nestler et al. 2007, Táncsics et al. 2010). Several members of the Rhodocyclaceae are known to degrade aromatic compounds under denitrifying conditions (Anders et al. 1995, Song et al. 1999, 2001, Zhou et al. 1995), while the presumable role of Rhodoferax-like species in the degradation of aromatic hydrocarbons is based on some recent studies (Martin et al. 2012, Aburto and Peimbert 2011).

The *Rhodoferax* clones recovered in the present study showed 100% 16S rRNA gene similarity to those detected in the most contaminated groundwater well of the SIReN site (UK) (Fahy et al. 2006), and 99.7–100% similarity to those detected in the benzene-contaminated aquifer of the Zeitz site (Germany, Alfreider and Vogt 2007).

The majority of *Azoarcus* clones recovered in the present study showed 100% 16S rRNA gene homology to *Azoarcus* sp. PbN1, similarly to that reported earlier (Táncsics et al. 2012).

The clones that were affiliated to an unclassified genus of the Rhodocyclaceae, and were predominant at the end of the monitoring period, clustered close to the genera *Uliginosibacterium*. In addition, identical environmental clones have been detected from the Casper contaminated site sediment, WY, USA (Callaghan et al. 2010), or Düsseldorf-Flingern site, Germany (Pilloni et al. 2011).

The second largest group of the community was affiliated to the Gammaproteobacteria, mainly to members of the genus *Pseudomonas*. The most abundant *Pseudomonas* clones showed 100% 16S rRNA gene similarity to the P. extremaustralis lineage. This bacteria is isolated form the Artic and known to degrade a wide variety of aromatic compounds and able to produce biofilms (Tribelli et al. 2012).

The other Proteobacter classes were not presented significantly in the groundwater samples.

Monitoring of the meta-cleavage dioxygenase gene expression showed that mRNA transcripts of subfamily I.2.C C23O and 3-IPC 2,3-dioxygenase genes were constantly detected. Regarding the subfamily I.2.C C23O genes, the newly designed degenerative primer set, designated as XYLE3, enabled the PCR amplification of a wide variety of sequences, even hitherto unknown types that could not be clearly assigned to any cultured bacterium. Our results also revealed that the metagenomics 3-IPC 2,3-dioxygenase genes described by Brennerova et al. (2009) were widely distributed and associated with BTEX-contaminated subsurface environments. Moreover, according to our best knowledge, this study is the first to report related mRNA transcripts from environmental samples. Regarding the detected subfamily I.2.A C23O mRNA transcripts, the presence of TOL-like plasmids harboring two homologous C23O genes can be speculated.

Since considerable diversity of subfamily I.2.C C23O mRNA transcripts was observed, and the clone libraries suggested changes in the expression profile of

the clusters detected, a SNuPE assay was developed in order to reveal these changes over the entire monitoring period. It was observed that only clusters B and C were detected constantly, and consequently their considerable role the microaerobic degradation of BTEX compounds can be hypothesized.
Accordingly, it can be assumed that cluster B was probably affiliated to an unclassified Rhodocyclaceae bacterium, while cluster C was most probably affiliated with a yet uncultured member of the genus *Rhodoferax* (Comamonadaceae).

Novel scientific result (Thesis I): The newly designed degenerative primer set, designated as XYLE3, enabled the PCR amplification of a wide variety of subfamily I.2.C C23O gene sequences, even hitherto unknown types that could not be clearly assigned to any cultured bacterium.

2.5 Stable isotope probing of hypoxic toluene degradation

Although the diversity of bacterial communities and subfamily I.2.C-type C23O gene pools at the Siklós site has been previously investigated (Táncsics et al. 2012, 2013), the affiliation of detected C23O genotypes and their possible role in hypoxic degradation processes remained unclear. The aim of this study was to address this by means of ^{13}C-labelling in combination with fingerprinting and sequencing of 16S rRNA and I.2.C-type C23O gene amplicons from SIP gradients. Toluene-degrading communities in site sediments were investigated at two time points of ^{13}C labelling. An overwhelming dominance of *Rhodocyclaceae*-related sequences was found in heavy DNA fractions. Especially, a *Quatrionicoccus*-related bacterium was thus identified as important hypoxic toluene degrader. The genus *Quatrionicoccus* contains only the type species *Q. australiensis*, which was isolated from activated sludge and is described as a strictly aerobic, Gram-negative coccus (Maszenan et al. 2002).

However, aromatic hydrocarbon degrading capability of the type strain has not been tested, and the type strain itself is currently not available in culture collections, unfortunately. The second most abundant labeled degrader lineage detected in heavy DNA was *Zoogloea* spp.. Members of this genus are primarily known for their floc-forming ability in sewage treatment plants, making them critical components of activated sludge processes. Within the genus, *Z. resiniphila* and *Z. oleivorans* have been described as degraders of petroleum hydrocarbons (Mohn et al. 1999, Farkas et al. 2015). The present study substantiates an important role of these aromatic hydrocarbon degraders in oxic or micro-oxic groundwater environments.

Elevated abundance of 16S rDNA sequences of a yet unaffiliated group of *Rhodocyclaceae*-related bacteria was also observed in the "heavy" DNA fractions. The closest relative of these bacteria is *Uliginosibacterium gangwonense* (~93% 16S rDNA similarity) and seem to be widely distributed in petroleum hydrocarbon contaminated subsurface environments. Their abundance in the Siklós groundwater was observed earlier and it was assumed that they encode subfamily I.2.C-type C23O gene and have a role in the hypoxic degradation of aromatic hydrocarbons. Interestingly, the C23O gene which was putatively linked to this group of bacteria earlier, was detected in the microcosms, and appeared as the second most dominant subfamily I.2.C-type C23O genotype in the "heavy" DNA fractions. This finding strengthens our previous assumption regarding the affiliation of this C23O genotype. However, this affiliation must be interpreted with caution in the lack of pure culture of these Rhodocyclaceae-related degraders.

Although the density-gradient ultracentrifugation of microcosm DNA clearly separated the "heavy" and "light" DNA fractions, a "medium" fraction was observable at both time points investigated. The separation of this fraction was caused by only one group of bacteria. The 16S rDNA amplicon pyrosequencing revealed that these bacteria belong to the genus *Rhodoferax* and their closest

relative is *Rhodoferax ferrireducens* (~96% 16S rDNA similarity). These bacteria are clearly associated to oxygen-limited or anaerobic petroleum hydrocarbon-contaminated subsurface environments (Callaghan et al. 2010, Táncsics et al. 2010, Aburto and Peimbert 2011, Táncsics et al. 2013, Larentis et al. 2013, Tischer et al. 2013). Results of the present study presume that these bacteria may also have role in the degradation of toluene, although they were not as apparently labeled as the previous lineages mentioned above.

The main unlabeled lineages detected in the microcosms were *Geobacter* spp. and *Azoarcus* spp.; some of them are usually reported as excellent anaerobic toluene degraders (Lueders 2017). Since oxygen was the only electron acceptor added to the microcosms their inactivity was expected. On the other hand the inactivity of *Pseudomonas* spp. can be surprising for the first sight by knowing that *P. putida* is one of the most widely utilized model organism for the study of aerobic toluene degradation (Martínez-Lavanchy et al., 2010). On the other hand, *Pseudomonas*-affiliated subfamily I.2.C-type C23O genes were absent from the community, which could have enabled these bacteria to take part in the degradation of toluene under hypoxic conditions.

However, it has to be noted that *Pseudoxanthomonas spadix* strains (which are capable of degrading all BTEX-compounds) usually harbor three subfamily I.2.C-type C23O genes in their genomes (Kim et al., 2008; Lee et al., 2012), and all of them were detectable in the microcosms of the present study, but always in the "light" DNA fractions such as the 16S rDNA of *Pseudoxanthomonas* spp.. Other, yet unidentified bacteria harboring subfamily I.2.C-type C23O gene also remained unlabeled during the experiment. The most dominant C23O genotype in the light fractions (with 802-bp T-RF) showed the largest similarity with metagenomic C23O clones which were isolated and investigated by Brennerova et al. (2009). It was shown that the enzyme coded by this C23O genotype prefers 3-methylcatechol as substrate which is an intermediate of aerobic toluene degradation. Nevertheless, bacteria harboring this C23O genotype did

not take part in the degradation of toluene in the microcosms. A possible explanation of this phenomenon is that these bacteria may prefer nitrate as electron-acceptor under oxygen-limited conditions while incorporating the available oxygen into the ring-structure of an aromatic hydrocarbon using oxygenases (Wilson et al., 1997). Due to the facts that nitrate was not added to the microcosms and that the Siklós aquifer was rather nitrate depleted such bacteria may have remained inactive during the experiment.

Novel scientific result (Thesis II): The results of stable isotope experiment clearly revealed the central role of *Zoogloea*, *Quatrionicoccus* and unknown Rhodocyclaceae bacteria in hypoxic toluene degradation at the center of the contaminant plume of the Siklós site. The aromatic hydrocarbon degrading ability of *Quatrionicoccus* genus was never described before.

2.6 Enrichment of dissimilatory Fe(III)-reducing bacteria

In essence, notable *Geobacter* and *Rhodoferax* populations and high diversity of *bssA* genes were found in the initial bacterial community. Surprisingly *Geobacter*-affiliated genes were not detected in the *bssA* clone library of the groundwater sample. The most closely related sequence type was a yet unidentified, most probably betaproteobacterial homologue.

The enrichment of *Rhodoferax*-related bacteria by using the four slightly different enrichment media was not succeeded, and subfamily I.2.C-type C23O genes were also not detectable in the enrichments. On the other hand, *Geobacter*-related bacteria became most dominant in three of the enrichments, and distinct *Geobacter* phylotypes were observable under different nitrogen availability. Nevertheless, only Betaproteobacterial, probably *Azoarcus*-affiliated *bssA* sequences were found even in the *Geobacter*-dominated enrichments. Accordingly, it is highly assumed that although a notable

Geobacter population can be observed at the Siklós BTEX-contaminated site, these bacteria may not play significant role in the anaerobic degradation of toluene in this environment.

Novel scientific result (Thesis III): Differential *Geobacter* enrichment media and conditions were established for the examined groundwater. It was verified that *Geobacter* species do not play significant role in the anaerobic degradation of toluene at the investigated site.

2.7 Describing a new bacterium species Zoogloea oleivorans sp. nov.

During our work a continuous attempt was made to enrich and isolate specific groups of microorganisms harboring aromatic hydrocarbon degrading enzymes in hypoxic conditions. In one case we isolated an unknown *Zoogloea* bacteria which possess subfamily I.2.C catechol 2,3-dioxygenases gene. The physiological, biochemical and phylogenetic data suggested that strain Buc^T represents a novel species, for which the name *Zoogloea oleivorans* was given.

Novel scientific result (Thesis IV): We isolated and described a novel *Zoogloea* species, *Zoogolea oleiovoran*s which possess subfamily I.2.C catechol 2,3-dioxygenases gene.

3. Conclusions, proposals

In summary, results of our studies clearly revealed the central role of *Zoogloea*, *Quatrionicoccus* and unknown Rhodocyclaceae bacteria in hypoxic toluene degradation at the center of the contaminant plume. Furthermore we isolated and describe a novel *Zoogloea* species which possess subfamily I.2.C catechol 2,3-dioxygenases gene. According to the literature these type gene could have enabled this bacteria to take part in the degradation of toluene under hypoxic conditions.

Our studies also revealed a notable diversity of this subfamily of catechol 2,3-dioxygenase (C23O) genes in the center of the contaminated well. Two of these C23O genotypes were statistically affiliated to an unclassified Rhodocyclaceae, and yet uncultured *Rhodoferax* bacteria (Comamonadaceae). To verify this presumptions an attempt was made to isolate the latter microorganism.

The Rhodoferax genus is really heterogenic we can find phototrophic, chemoorganotrophic, fermenting and Fe(III)- reducing members also. During the enrichments the Fe(III)-reducing capability of the bacteria was presumed, but unfortunately the isolation was failed. Henceforth we are going to develop new enrichment technics by utilizing the phototrophic property of the genus.

It also has to be noted that some I.2.C-type C23O genes (for example *Pseudoxanthomonas spadix* affiliated ones) remains inactive despite of the hypoxic conditions. The most dominant C23O genotype in the light fractions (802-bp T-RF) showed high similarity with metagenomic C23O clones reported by Brennerova et al. (2009). Functional genomics showed that the enzyme coded by this C23O genotype prefers 3-methylcatechol as substrate, an intermediate of aerobic toluene degradation. Nevertheless, bacteria harboring this C23O genotype were not labeled in our SIP microcosms. It is possible to speculate that these degraders could actually prefer nitrate as electron-acceptor under hypoxic conditions, while utilizing available oxygen for catabolic

oxygenases (Wilson et al., 1997). since we did not add nitrate to the microcosms, and the fact the Siklós site is depleted in nitrate (Táncsics et al., 2013), such degraders may have remained inactive during our experiment.

The role of the *Pseudomonas* genus in a microaerobic BTEX contaminated environment is also questionable. However these bacteria were active at the beginning of the monitoring period during SIP incubation remained unlabeled. On the other hand, *Pseudomonas*-affiliated subfamily I.2.C-type C23O genes, which could have enabled these bacteria to take part in the degradation of toluene under hypoxic conditions, were only detectable in 2010.

We show that identified microaerobic toluene degraders mostly harbored subfamily I.2.C-type C23O genes, which may be of crucial importance for the degradation of aromatic hydrocarbons under oxygen-limited conditions. However, not all C23O genotypes were actually ^{13}C-labelled, suggesting that ecophysiological fine-tuning, rather than catabolic repertoire contributes to niche definition amongst hypoxic degraders of BTEX compounds in groundwater systems.

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