

## RESEARCH ARTICLE

# Stable isotope probing of hypoxic toluene degradation at the Siklós aquifer reveals prominent role of *Rhodocyclaceae*

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**One sentence summary:** A combination of 16S rRNA gene amplicon sequencing and T-RFLP fingerprinting of C23O genes from SIP gradient fractions revealed the central role of degraders within the *Rhodocyclaceae* in hypoxic toluene degradation.

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## ABSTRACT

The availability of oxygen is often a limiting factor for the degradation of aromatic hydrocarbons in subsurface environments. However, while both aerobic and anaerobic degraders have been intensively studied, degradation betwixt, under micro- or hypoxic conditions has rarely been addressed. It is speculated that in environments with limited, but sustained oxygen supply, such as in the vicinity of groundwater monitoring wells, hypoxic degradation may take place. A large diversity of subfamily I.2.C extradiol dioxygenase genes has been previously detected in a BTEX-contaminated aquifer in Hungary. Older literature suggests that such catabolic potentials could be associated to hypoxic degradation. Bacterial communities dominated by members of the *Rhodocyclaceae* were found, but the majority of the detected C23O genotypes could not be affiliated to any known bacterial degrader lineages. To address this, a stable isotope probing (SIP) incubation of site sediments with <sup>13</sup>C<sub>7</sub>-toluene was performed under microoxic conditions. A combination of 16S rRNA gene amplicon sequencing and T-RFLP fingerprinting of C23O genes from SIP gradient fractions revealed the central role of degraders within the *Rhodocyclaceae* in hypoxic toluene degradation. The main assimilators of <sup>13</sup>C were identified as members of the genera *Quatronicoccus* and *Zoogloea*, and a yet uncultured group of the *Rhodocyclaceae*.

**Keywords:** biodegradation; oxygen limitation; DNA-stable isotope probing; subfamily I.2.C extradiol dioxygenase (C23O); groundwater; *Rhodocyclaceae*

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## INTRODUCTION

The contribution of microbes to the removal of BTEX compounds (benzene, toluene, ethylbenzene and xylenes) from groundwater ecosystems has been intensively investigated over the last decades (Lueders 2017). However, most studies have addressed either strictly aerobic or anaerobic degradation and degraders, often by using enriched or pure cultures in highly artificial laboratory systems. In subsurface ecosystems, the availability of oxygen is often restricted, with hydrocarbon contamination causing microoxic or anoxic conditions even in shallow aquifers. Under oxic conditions, genes for aromatic ring-cleavage dioxygenase enzymes are key to the degradation of monoaromatic compounds (El-Naas, Acio and El Telib 2014). Aerobic degraders use oxygen not only for respiration but also as a cosubstrate for these enzymes. However, Kukor and Olsen (1996) suggested that a specific group of extradiol dioxygenases (subfamily I.2.C) was adapted to environments with low oxygen concentrations, hinting at their role in ring-cleavage reactions in what they called “oxygen-requiring, but nitrate-enhanced” hypoxic degradation. Nevertheless, it has to be noted that ring-cleaving dioxygenases belonging to the same subfamily may show different oxygen affinities as this was observed in case of chlorocatechol 1,2-dioxygenases (Balcke et al. 2008).

Previous investigations of an oxygen-limited BTEX-contaminated shallow aquifer in Siklós, Hungary have revealed a notable diversity of catechol 2,3-dioxygenase (C23O) genes encoding subfamily I.2.C-type extradiol dioxygenases at the site (Táncsics et al. 2012, 2013). It was also shown that the bacterial community at this site was dominated by microorganisms affiliated to the *Comamonadaceae* and *Rhodocyclaceae*. Both betaproteobacterial lineages are known to harbor aromatic hydrocarbon degraders. However, *Comamonadaceae*-affiliated degraders (e.g. members of the genera *Acidovorax*, *Comamonas*, *Delftia*, *Diaphorobacter*, *Hydrogenophaga*, *Polaromonas* and *Variovorax*) utilize BTEX-compounds only aerobically and usually harbor subfamily I.2.C-type C23Os (Parales 2010). On the other hand, many *Rhodocyclaceae*-affiliated degraders degrade aromatic hydrocarbons under anaerobic conditions (members of the genera *Azoarcus*, *Dechloromonas* and *Thauera*) (Weelink, van Eekert and Stams 2010). Recently, however, some members of the genus *Zoogloea* and the type species of the genus *Rugosibacter* have been identified as aerobic hydrocarbon degraders (Jechalke et al. 2013; Farkas et al. 2015; Corteselli, Aitken and Singleton 2017), showing that members of the *Rhodocyclaceae* can also have a role in aerobic degradation processes.

Although I.2.C-type C23O genes can be abundant in hypoxic BTEX-contaminated groundwater ecosystems (Táncsics et al. 2012; Benedek et al. 2016), the majority of these genotypes cannot yet be linked to cultured bacteria. The aim of the present study was to identify, by means of DNA stable isotope probing (DNA-SIP), key degraders and associated I.2.C-type C23O genes active in toluene degradation under oxygen-limited conditions. For this, fresh sediment samples taken from the bottom of a monitoring well in the center of the BTEX plume at the Siklós site were incubated in microcosms under amendment of  $^{13}\text{C}_7$ -toluene and a repeated replenishment of <0.5 mg/l oxygen as electron acceptor and as co-substrate for aromatic-ring-hydroxylating and ring-cleaving dioxygenases. Key bacteria labelled during microaerobic toluene degradation were identified as members of the *Rhodocyclaceae* and their catabolic genotypes were unraveled. This study provides new evidence that the known diversity of hypoxic degraders of BTEX compounds is still incomplete.

## MATERIALS AND METHODS

### Sampling site and sample acquisition

Sampling was performed at an intensively studied BTEX-contaminated aquifer (Táncsics et al. 2012, 2013; Farkas et al. 2017) in Siklós, Hungary, in April 2015. Sediment samples were taken from the bottom of a monitoring well at 6 m below ground surface in the center of the contaminant plume (well ST-2). Well sludge and hypoxic groundwater was retrieved by suction pumping (Gardena, Ulm, Germany) into a clean 10-L plastic jerrycan. After settling for ~20 min, sediment sludge was dispensed into sterile 1-L glass bottles filled with *in situ* groundwater to minimize atmospheric exposure and transported to the laboratory under cooling.

### Incubation of sediments

Triplicates of 5g<sub>ww</sub> homogeneously mixed sediment material were transferred into sterile 100-mL serum bottles containing 50 mL of artificial groundwater medium (Winderl et al. 2010). To increase microbial activity 5 μm cAMP was added to the medium (Bruns, Cypionka and Overmann 2002). Bottles were sparged aseptically with N<sub>2</sub>/CO<sub>2</sub> (80:20, v/v) for 10 min, after which the desired volume of sterile (0.2 μm-pore-size-filtered) air was injected into the bottles through gastight viton rubber stoppers. 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. Oxygen was replenished once every 24 h. A 5 μL of either non-labeled ( $^{12}\text{C}$ ) or fully labeled ( $^{13}\text{C}_7$ ) toluene (Sigma-Aldrich, St. Louis, MO, USA) were injected to the microcosms. Abiotic control bottles (autoclaved three times) amended with unlabelled toluene were also prepared to exclude abiotic toluene loss or redox reactions. The bottles were incubated at 16°C in a rotary shaker at 145 rpm for over 7 d.

### Process measurements

The concentration of dissolved oxygen in the liquid phase of the microcosms was measured by using planar oxygen sensor spots and a Fibox 3 Oxygen Meter (PreSens, Regensburg, Germany). At each sampling spot, dissolved oxygen concentrations were registered every second during 1 min, and the results were displayed by using the OxyView-PST3 software (V7.01, PreSens). Toluene concentrations were determined by headspace analysis on an ISQ Single Quadrupole GC-MS (Thermo Fischer Scientific, Waltham, MA, USA) via a SLB-5ms fused silica capillary column (Sigma-Aldrich). The oven temperature was set to 40°C for 3 min, then ramped at a rate of 20°C/min to 190°C, and held for 1 min. The mass spectrometer (MS) was operated at 250°C in full scan mode.

### Nucleic acid extraction and ultracentrifugation

Sediments were collected from sacrificed microcosms after 3 and 7 d of incubation by centrifugation at 2360 g at 4°C for 10 min using a Rotanta 460 R (Hettich, Tuttlingen, Germany). Sludge pellets were frozen immediately at –80°C and DNA was extracted by using the RNA PowerSoil Total RNA Isolation Kit (MoBio, Carlsbad, CA, USA) in combination with the RNA PowerSoil DNA Elution Accessory Kit (MoBio). DNA samples were stored frozen at –80°C until downstream analyses. Approximately 1 μg of Qubit-quantified (Invitrogen, Paisley, UK) DNA extract was loaded onto a gradient medium of CsCl (average

density 1.71 g/mL, Calbiochem, Darmstadt, Germany) in gradient buffer (0.1 M Tris-HCl at pH 8, 0.1 M KCl, 1mM EDTA) and centrifuged (180 000 g, ~68 h) as previously described (Lueders 2015). A total of 12 fractions from each gradient were collected from 'heavy' to 'light' using a Perfusor V syringe pump (B. Braun, Melsungen, Germany). Refractometric measurement of fraction buoyant densities (BD) and the recovery of DNA from gradient fractions were performed as described (Lueders 2015).

### qPCR, T-RFLP fingerprinting and amplicon sequencing

DNA samples recovered from the CsCl gradient fractions were analyzed by qPCR targeting bacterial 16S rRNA gene as described (Kunapuli, Lueders and Meckenstock 2007; Pilloni et al. 2011). Eight DNA fractions (from 3rd to 10th) of each gradient were selected for bacterial 16S rRNA gene-targeted terminal restriction fragment length polymorphism (T-RFLP) fingerprinting, together with total DNA extracts of the inoculum. FAM labeled amplicons were generated with the primers Ba27f (5'-FAM-AGA GTT TGA TCM TGG CTC AG-3') and 907r (5'-CCG-TCA-ATT-CCT-TTG-AGT-TT-3') similarly as described earlier (Pilloni et al. 2011). Amplicons were restricted using *RsaI*, separated by capillary electrophoresis and electropherograms were evaluated as reported (Pilloni et al. 2011).

DNA extracts were also subjected to I.2.C-type C23O gene T-RFLP fingerprinting. VIC labeled amplicons were generated with the primers XYLE3F (5'-VIC- TGY TGG GAY GAR TGG GAY AA-3') and XYLE3R (5'-TCA SGT RTA SAC ITC SGT RAA-3') in a ProFlex PCR System (Life Technologies, Carlsbad, CA, USA) applying cycling conditions and PCR chemistry as reported (Táncsics et al. 2013). Amplicons were digested with *AluI*, then electropherograms were generated and analyzed as described earlier (Farkas et al. 2017).

Non-density-resolved total DNA extracts from the inoculum and selected gradient fractions were also subjected to 16S rDNA amplicon pyrosequencing.

Bacterial 16S rRNA gene amplicon pyrosequencing was performed using a unidirectional sequencing approach as described (Zhang and Lueders 2017). Barcoded amplicons for multiplexing were prepared using the primers Ba27f (5'-aga gtt tga tcm tgg ctc ag-3') and Ba907r (5'-ccg tca att cmt ttr agt t-3') extended with the respective Lib-L adapters, key sequence and a multiplex identifier (MID) attached to the forward primer as recommended for the 454 GS FLX+ protocol (Roche, Basel, Switzerland). PCR amplification conditions were the same as described before (Karwautz and Lueders 2014). Amplicons were visualized with gel electrophoresis in a 1.5% agarose gel. Cleanup of the amplicons was done with a PCRextract kit (5Prime, Hamburg, Germany) according to the manufacturer's protocol. Quality of single amplicons was checked for primer dimer contamination and correct fragment size using the Bioanalyzer2100 (Agilent, Santa Clara, CA, USA) loading High Sensitivity DNA assay chips (Agilent), as described by the manufacturer. One multiplexed amplicon pool (consisting of 20 amplicon libraries) was prepared in equimolar amounts ( $5 \times 10^9$  molecules  $\mu\text{l}^{-1}$ ) of barcoded amplicons as quantified by the Quant-iT PicoGreen dsDNA quantification kit (Invitrogen). The amplicon pool then underwent a second purification step with Agencourt AMPure-XP beads (Beckman Coulter, Brea, CA, USA) using an adapted heat-denaturation protocol (Roche). Emulsion PCR and emulsion breaking were performed following protocols of Roche and pyrosequencing was performed on a 454 GS FLX+ sequencer by IMG/M Laboratories, Planegg, Germany.

### Analysis of sequencing data

Initial quality filtering of the raw pyrosequencing reads was done by using the automated amplicon pipeline of the GS Run Processor with the LongAmplicon3 filter (Roche). Sequences were then de-multiplexed to separate MID barcodes (Pilloni et al. 2012), initial quality trimming was done in GREENGENES; using the TRIM function with the default settings (DeSantis et al. 2006). Trimmed sequences were uploaded and analyzed via the NGS analysis pipeline of the SILVA rRNA gene database project (SILVAngs 1.3) (Quast et al. 2013). Reads were aligned using the SILVA Incremental Aligner (SINA SINA v1.2.10 for ARB SVN (revision 21008)) (Pruesse, Peplies and Glöckner 2012) against the SILVA SSU rRNA SEED and quality controlled (Quast et al. 2013). Reads shorter than 50 aligned nucleotides or below 40 alignment score, reads with more than 2% of ambiguities or more than 2% of homopolymers were excluded from the downstream processing. Dereplication and clustering of the unique reads into operational taxonomic units (OTUs) was done by using cd-hit-est (version 3.1.2) (Li and Godzik 2006) running in accurate mode, ignoring overhangs and applying identity criteria of 1.00 and 0.98, respectively. The classification of the OTUs was performed by a local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 123; <http://www.arb-silva.de>) using blastn (version 2.2.30) with standard settings (Camacho et al. 2009). Weak BLAST hits (below 93%) or reads without any BLAST hits remained unclassified and were assigned to the metagroup "No Relative". For downstream data handling, relative abundances were selected from the SILVAngs pipeline output. OTUs with less than 1% relative abundance were summarized in a composite "<1%" group. Selected amplicon contigs have been deposited at GenBank under the accession numbers KY499472 to KY499476. All sequencing read raw data are deposited at the SRA under the project accession numbers SAMN07673532-SAMN07673540.

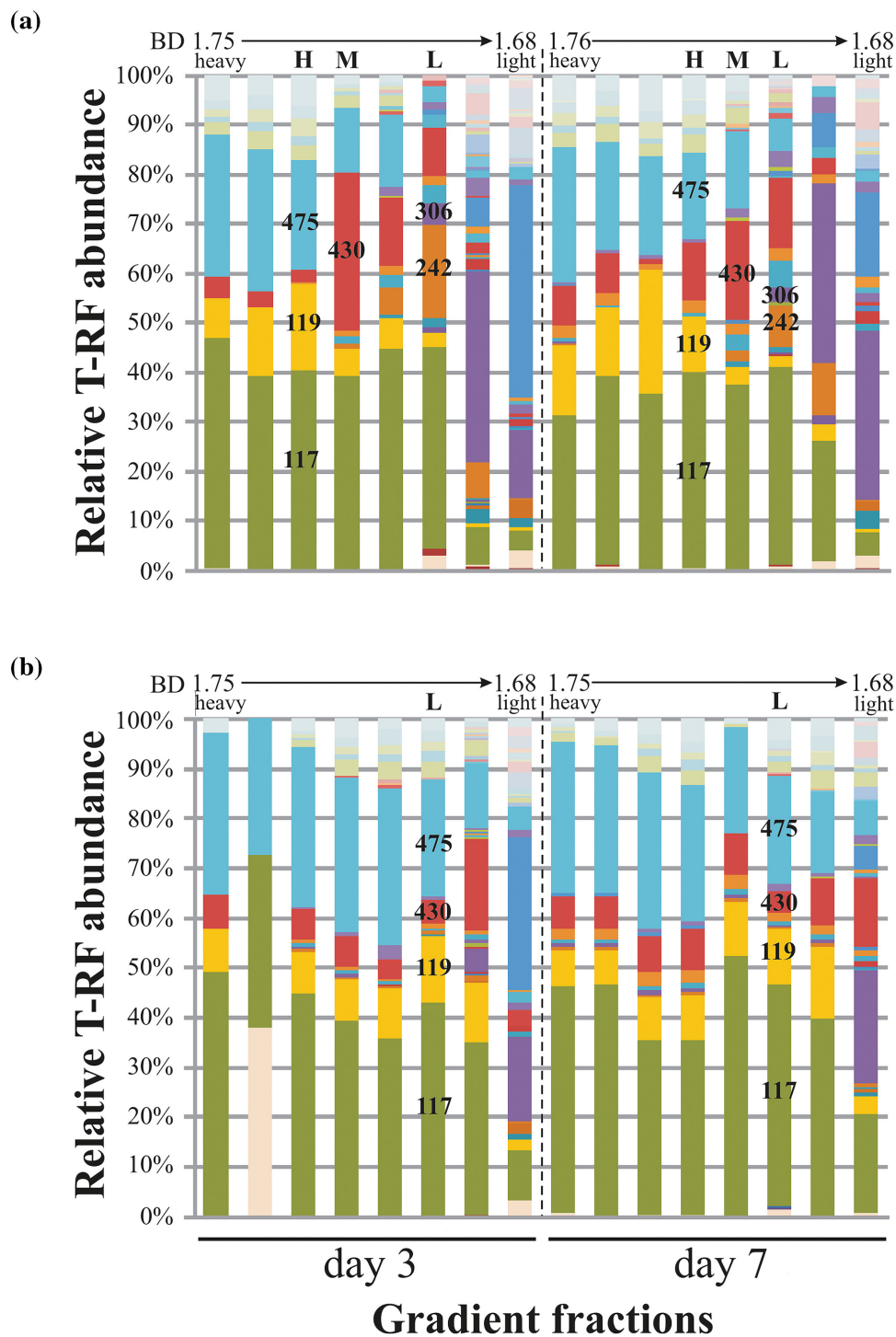
### Cloning, sequencing and phylogenetic analysis

C23O amplicons generated with the primer set XYLE3F/XYLE3R were cloned and sequenced (Táncsics et al. 2013) from the initial sediment sample, as well as from selected "heavy" and "light" DNA fractions of the day 3  $^{13}\text{C}$ -toluene SIP gradient. Selected terminal restriction fragments (T-RFs) predicted *in silico* for representative clones were verified *in vitro*. Phylogenetic trees were reconstructed from sequence data using neighbor-joining as described (Táncsics et al. 2013). Sequences generated by cloning were deposited with GenBank and can be found under the accession numbers KY440386 – KY440395.

## RESULTS

### Exposure of sediments to $^{13}\text{C}$ -toluene

Rapid depletion of toluene was observed in all enrichments (Fig. S1, Supporting Information) under simultaneous consumption of oxygen (data not shown). Roughly 70% of the toluene was depleted from the biotic enrichments after 3 d of incubation, while its concentration was under the detection limit by the seventh day of incubation (Fig. S1, Supporting Information). The abiotic loss from control incubations was marginal. Enrichments incubated for seven days received ~7.8 mL of oxygen during the incubation. According to Wiedemeier et al. (1999) this amount of oxygen may be sufficient for the complete removal of  $4.7 \times 10^{-5}$  mol toluene (a concentration of ~1 mM) present

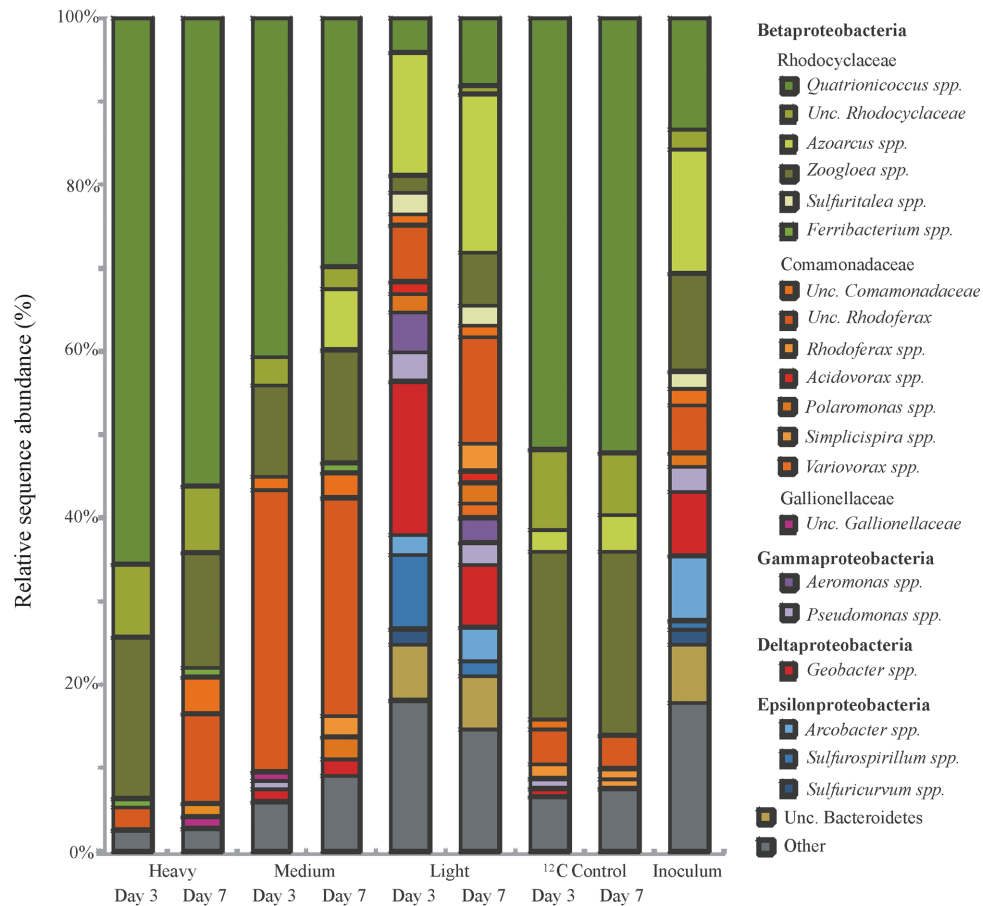


**Figure 1.** Abundance of bacterial 16S rRNA gene T-RFs across density-resolved gradient fractions of DNA from enrichments amended with (A)  $^{13}\text{C}$  or (B)  $^{12}\text{C}$ -toluene. Representative heavy (H), medium (M) and light (L) DNA fractions were selected and subjected to amplicon sequencing. Identified characteristic T-RFs (bp) mentioned in the text are indicated. BD, CsCl buoyant density ( $\text{g mL}^{-1}$ ).

in the enrichments through biodegradation. Accordingly, consumption of oxygen considerably slowed by the end of the experiment, when toluene was depleted, as the oxygen injected on the 6th day of incubation was not completely consumed a day later (data not shown).

#### Identification of labeled bacteria

Two time points were selected for the detection of labeled DNA by isopycnic centrifugation of extracts from single microcosms: day 3, where considerable degradation activity was suggested, and day 7, after toluene was depleted in the enrichments. At



**Figure 2.** Relative read abundance of major taxa in bacterial pyrotag libraries of heavy, medium and light DNA fractions of  $^{13}\text{C}$ -toluene SIP gradients, representative light fractions of  $^{12}\text{C}$ -control gradients and the initial sediment inoculum. All genera contributing more than 1% abundance were depicted.

both time points, clear shifts in buoyant density (BD) compared to respective  $^{12}\text{C}$ -control DNA was observed in SIP gradients (Fig. S2, Supporting Information). Bacterial 16S rRNA gene-targeted T-RFLP fingerprinting of density resolved DNA detected clear distinctions between heavy and light DNA fractions of  $^{13}\text{C}$ -gradients (Fig. 1). Heavy fractions of DNA from  $^{13}\text{C}$ -toluene sediments showed a dominance of the 117-, 119- and 475-bp T-RFs. Light DNA fractions were enriched in the 242- and 306-bp T-RFs, while the 117 bp T-RF was also abundant here. In-between, medium BD fractions showed a selection of the 430-bp T-RF, giving a distinct community pattern between heavy and light fractions. DNA fractions from  $^{12}\text{C}$ -control gradients were more similar over the entire BD range and were dominated mainly by two T-RFs: the 117- and 475-bp fragments.

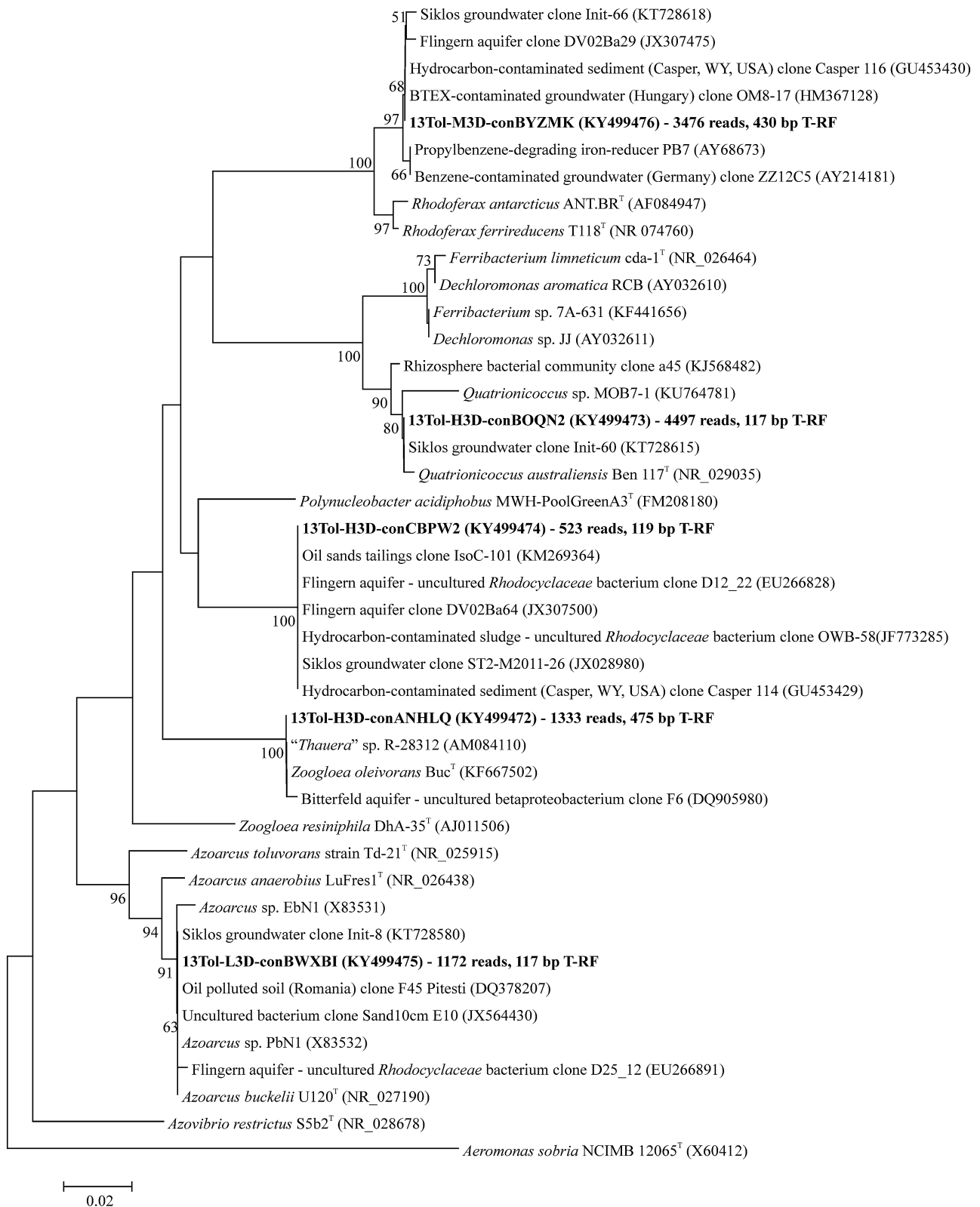
Bacterial 16S rRNA gene amplicons were sequenced from heavy, medium and light gradient fractions of  $^{13}\text{C}$ -gradients at both time points, as well as for  $^{12}\text{C}$ -control gradients and non-density resolved DNA from the initial sediment (Fig. 2, Table S1, Supporting Information). Libraries from heavy DNA after 3 and 7 d appeared especially enriched in reads affiliated to *Quatronicoccus* spp., *Zoogloea* spp., as well as other uncultured *Rhodocyclaceae*. In combination with T-RFs previously reported for bacterial rRNA genes from the same site (Táncsics et al. 2013) the affiliation of T-RFs detected across gradients was thus possible (Fig. 3). The 117-bp T-RF in the “heavy” fractions represented amplicons affiliated to *Quatronicoccus* spp. (up to ~60% read abundance in heavy DNA). The 119-bp and 475-bp T-RFs were linked to *Zoogloea* spp. and a yet uncultured member of the

*Rhodocyclaceae*, respectively. In contrast, sequences represented by the 117-bp T-RF in the light fractions appeared mainly affiliated to *Azoarcus* spp. Thus, 16S rRNA gene sequencing resolved labeled and unlabeled bacterial populations apparently represented by the same (117-bp) T-RF. Besides, the 242- and 306-bp T-RFs detected in light fractions represented amplicons affiliated to *Geobacter* spp. and the *Bacteroidetes*. The 430-bp T-RF enriched in intermediate fractions represented reads related to *Rhodoferrax* spp.

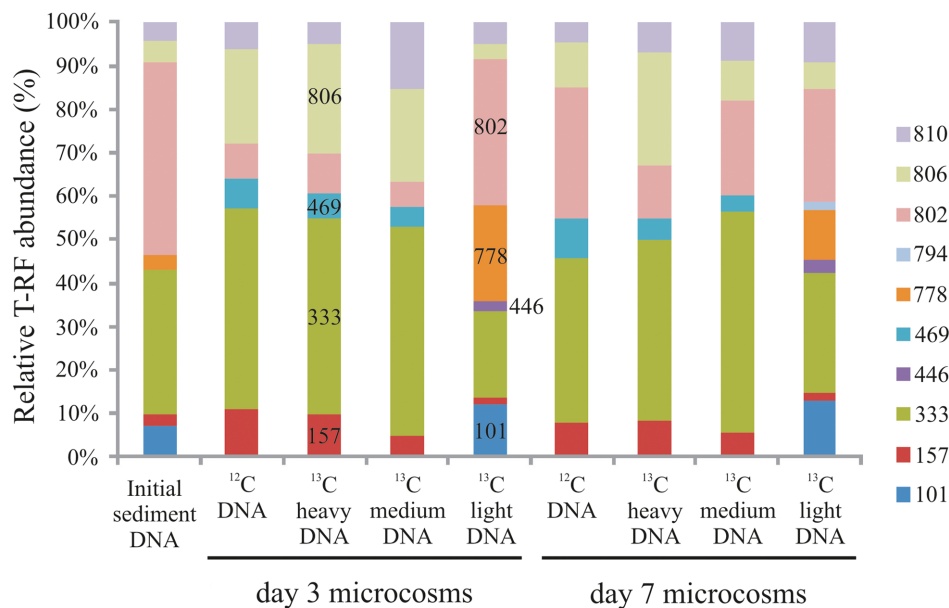
Furthermore, amplicon sequencing revealed that intermediate DNA fractions were still highly dominated by *Rhodocyclaceae*, but reads within the *Comamonadaceae* were also observed, mostly affiliated to the yet uncultured lineage of genus *Rhodoferrax*. In the light DNA fractions the abundance of reads within the *Betaproteobacteria* decreased while sequences affiliated to the *Gammaproteobacteria* (*Aeromonas* and *Pseudomonas* spp.), *Deltaproteobacteria* (*Geobacter* spp.), *Epsilonproteobacteria* (*Arcobacter* and *Sulfurospirillum* spp.) as well as *Bacteroidetes* consistently became more abundant in both  $^{13}\text{C}$ -gradients (Fig. 2, Table S1, Supporting Information).

### Subfamily I.2.C-type C230 genes detected in SIP gradient fractions

The diversity of I.2.C-type C230 genes at the Siklós site has been investigated previously (Táncsics et al. 2012, 2013). However, the majority of the genotypes detected could not be affiliated to known bacterial degraders of BTEX compounds at that time. To



**Figure 3.** Phylogenetic placement of selected assembled OTU-level sequencing contigs (given in bold) of bacterial 16S rRNA gene amplicons from the SIP microcosms after 3 d. Contig naming indicates the DNA fraction (H - heavy, M - medium, L - light) as well as the incubation period (3D). Comprised total reads and predicted T-RFs (bp) are also indicated. T-RFs were predicted from sequence data, but are given as T-RFs actually measured in electropherograms, as first verified by Tánácsics (2013). Bacterial lineages identified as key toluene degraders in SIP are highlighted in grey. GenBank accession numbers are also indicated. The tree was constructed using the neighbor-joining methods with Kimura's two-parameter calculation model. Bootstrap values are shown as percentages of 1000 replicates; only values over 50% are shown. The 16S rDNA sequence of the gammaproteobacterium *Aeromonas sobria* (detected in the "light" DNA fractions) was used as outgroup. Scale bar, 0.02 substitutions per nucleotide position.



**Figure 4.** T-RFLP fingerprinting of subfamily I.2.C-type C23O genotypes from heavy, medium and light DNA fractions of  $^{13}\text{C}$ -toluene SIP gradients, representative light fractions of  $^{12}\text{C}$ -control gradients and the initial sediment sample. Characteristic T-RFs (bp) mentioned in the text are indicated.

address this, a T-RFLP fingerprinting assay targeting I.2.C-type C23O genes was applied to screen density resolved C23O genotypes. In contrast to 16S rRNA gene targeted T-RFLP fingerprinting, heavy and intermediate DNA fractions showed similar C23O fingerprints (Fig. 4). Dominant C23O T-RFs in heavy fractions were the 333- and 806-bp T-RFs at both time points, of which the first was also highly abundant in the inoculum. Further minor T-RFs (157- and 469-bp) were also enriched in heavy fractions. The dominant T-RF in the light fractions was at 802-bp, highly abundant also in the initial inoculum. Furthermore, C23O T-RFs at 778-, 101- and 446-bp were exclusively detectable in light DNA.

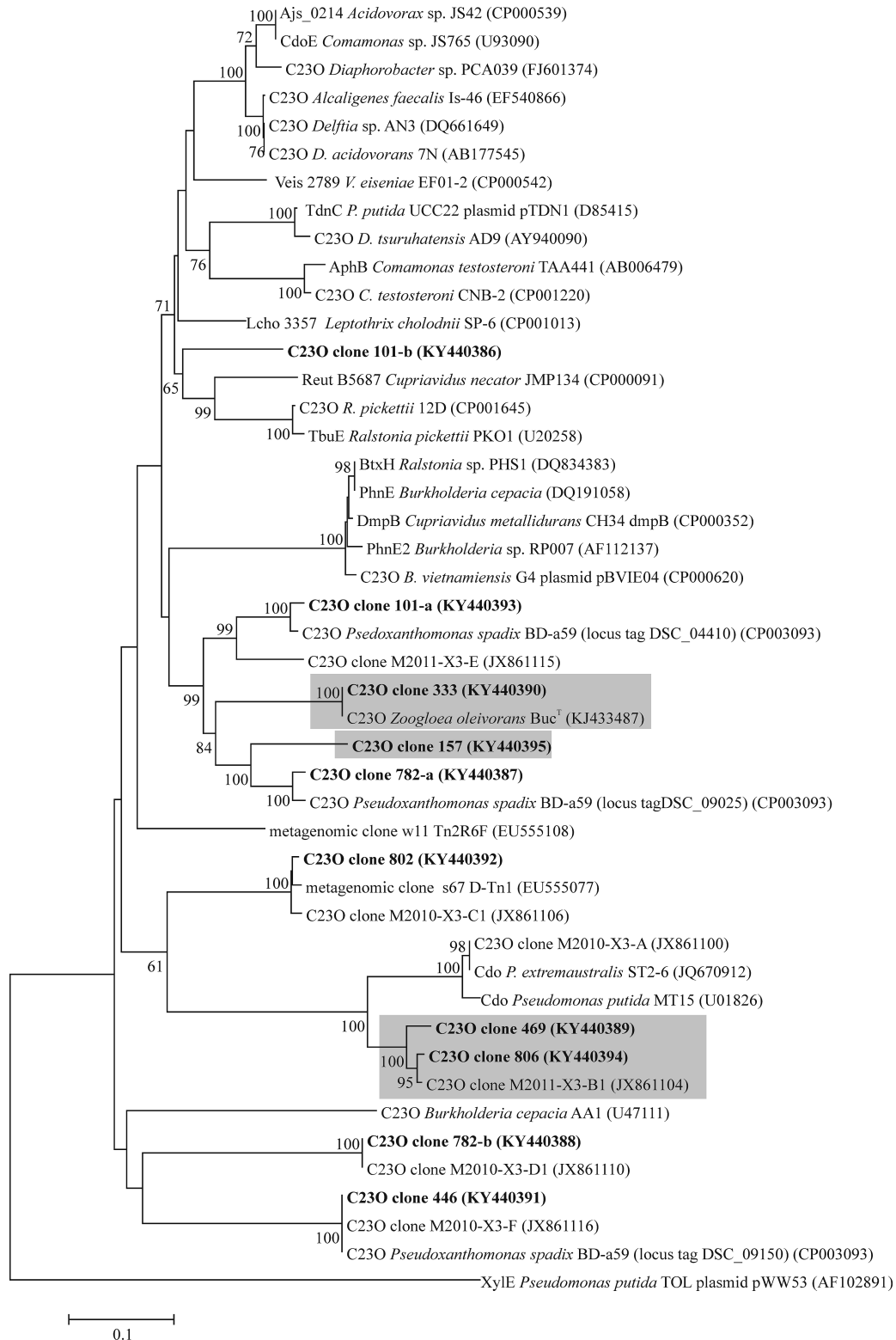
To identify these T-RFs, clone libraries of C23O amplicons were generated and sequenced from the inoculum, as well as heavy and light DNA fractions of the day 3 microcosm. Thus, the 333-bp T-RF represented known C23O genes of *Zoogloea oleivorans* (Fig. 5), while the 806-bp T-RF represented a yet unaffiliated C23O genotype with low similarity (86% at the nucleotide level) to the *cdo* gene of *P. putida* MT15. Other minor T-RFs in heavy fractions represented unaffiliated C23O genes as well. In contrast, the 802-bp T-RF dominating the C23O gene pool in light DNA fractions, represented sequence types with high similarity (99%) to a yet unaffiliated, but heterologously characterized C23O gene (Brennerova et al. 2009). Furthermore, the 101-, 446- and 778-bp T-RFs represented three I.2.C-type C23O genes of *Pseudoxanthomonas spadix*. However, other amplicons related to yet unaffiliated C23O genes were also comprised in these fingerprinting peaks.

## DISCUSSION

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 oxic or hypoxic degradation processes remained unclear. The aim of this study was to address this by means of  $^{13}\text{C}$ -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  $^{13}\text{C}$  labelling. A 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). The high abundance of this bacterium in the groundwater of the Siklós site has been noted earlier (Farkas et al. 2017). However, aromatic hydrocarbon degrading capability of the type strain has not been tested, and it is currently not available in culture collections. The two most closely related genera of *Quatrionicoccus* are *Ferribacterium* and *Dechloromonas* spp., the latter including *Dechloromonas aromatica*, a well-investigated aromatic hydrocarbon degrader (Coates et al. 2001). This is reported to degrade aromatic hydrocarbons using a dioxygenase-based pathway (not subfamily I.2.C C23O-based) under respiration of oxygen, chlorate or nitrate, giving rise to speculations about cryptic catabolic pathways at the interphase of aerobic and anaerobic metabolism (Salinero et al. 2009; Weelink, van Eekert and Stams 2010; Lueders 2017).

The second most abundant labeled degrader lineage detected in heavy DNA was *Zoogloea* spp. Screening of subfamily I.2.C C23O genes across gradient fractions indicated consistent labelling of *meta*-cleavage pathway encoding genes affiliated to *Zoogloea*. Members of this genus are primarily known for their floc-forming ability in sewage treatment plants, making them critical components of activated sludge processes (Shao et al. 2009). Within the genus, *Z. resiniphila* and *Z. oleivorans* have been described as degraders of petroleum hydrocarbons (Farkas et al. 2015; Monh et al. 1999). Jechalke et al. (2013) has investigated benzene degradation by a biofilm community in an aerated groundwater treatment pond. rRNA-SIP revealed a prominent role of *Zoogloea*-related degraders in the system. The present study substantiates an important role of these aromatic hydrocarbon degraders in oxic or micro-oxic groundwater environments.



**Figure 5.** Neighbor-joining tree showing the phylogenetic placement of subfamily I.2.C-type C23O gene clones retrieved from the initial sediment DNA, heavy and light DNA fractions of day 3  $^{13}\text{C}$ -toluene SIP gradient. Clones from this study are in bold, GenBank accession numbers are indicated. Clone naming includes measured T-RF lengths (*AluI* digestion). Clones dominantly or exclusively found in the heavy DNA fractions are highlighted in grey. Bootstrap values are shown as percentages of 1000 replicates; only values over 50% are shown. The subfamily I.2.A-type C23O gene of TOL plasmid pWW53 was used as outgroup. Scale bar, 0.1 substitutions per nucleotide position.



The second most abundant C23O genotype detected in heavy DNA was the as-yet unidentified catabolic gene lineage represented by the 806 bp T-RF. The high abundance and marked enrichment of this gene in  $^{13}\text{C}$ -labelled DNA suggests that it could be affiliated to one of the dominating degraders identified in labelled 16S rRNA genes. It is tempting to speculate that this C23O genotype could actually be hosted by the *Quatronicoccus*-relatives, however also other scenarios cannot be excluded, since degradation of toluene by these bacteria must not essentially involve catabolic pathways via C23O. Also, we have previously tentatively affiliated (Táncsics et al. 2013) the 806-bp T-RF C23O phylotype to the yet unidentified *Rhodocyclaceae*-related 16S sequences which were also found, albeit at much lower abundance, in heavy DNA fractions. The closest relative of these bacteria is *Polynucleobacter acidiphobus* (~95.5% 16S rDNA similarity). However, as long as isolates of either of these *Rhodocyclaceae*-related degraders or of *Quatronicoccus* spp. are not available, these interpretations must clearly be cautioned. Alternatively, metagenomics of single-cell approaches (Blainey 2013; Rinke et al. 2014) may also help to resolve this dilemma.

Besides the abundant peaks of fully  $^{13}\text{C}$ -labelled DNA detected in heavy gradient fractions, a distinct community was also observed in intermediate gradient fractions. Here, 16S rRNA reads of the genus *Rhodoferax* were consistently enriched, *Rhodoferax ferrireducens* being their closest relative (~96% 16S rDNA similarity). These bacteria have been frequently reported from oxygen-limited or anaerobic subsurface environments contaminated with petroleum hydrocarbons (Callaghan et al. 2010; Aburto and Peimbert 2011; Táncsics et al. 2010, 2013; Larentis, Hoermann and Lueders 2013; Tischer et al. 2013). Moreover, previous SIP studies have indicated a role of this lineage in the aerobic degradation of phenantrene and naphthalene (Jeon et al. 2003; Martin et al. 2012). Results of our present study suggest that these bacteria may also have a role in the degradation of toluene, although labeling was not as apparent as for other dominating degraders. This could potentially be explained by the fact that certain *Rhodoferax* species grow very slowly (Kaden et al. 2014), and it was shown that *R. ferrireducens* is more adapted for high growth yields than rapid growth (Zhuang et al. 2011).

The main unlabeled lineages detected in the microcosms were affiliated to *Geobacter* and *Azoarcus* spp. Members of both genera are well known as anaerobic toluene degraders (Lueders 2017). While both may have originally been active in deeper oxygen-limited sediments at the site, they were clearly not active in our hypoxic microcosms. More surprisingly, reads affiliated to *Pseudomonas* spp. also remained unlabeled during SIP incubation. *P. putida* is one of the most widely utilized model organisms 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, which could have enabled these bacteria to take part in the degradation of toluene under hypoxic conditions (Kukor and Olsen 1996), were not detected in the Siklós samples. It also has to be noted that *Pseudoxanthomonas spadix* (capable of degrading all BTEX-compounds) usually harbors three subfamily I.2.C-type C23O genes in its genome (Kim et al. 2008; Lee et al. 2012). All of them were detectable, but remained unlabeled in our study, just like the 16S rRNA genes of *Pseudoxanthomonas* spp. Nevertheless, toluene concentration in the microcosms was ~1 mM, which can be toxic even for some toluene-degrading bacteria (Rabus et al. 1993) and could cause their inactivity as well.

The most dominant C23O genotype in the light fractions (802-bp T-RF) showed high similarity with metagenomic C23O

clones retrieved by Brennerova et al. (2009) from jet-fuel contaminated soil. Functional genomics showed that the enzyme coded by this C23O genotype preferred 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 and Bouwer 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.

In summary, this study shows that a notable diversity of degraders within the *Rhodocyclaceae* is active in hypoxic toluene degradation in sediments from the Siklós site. This includes previously unidentified degraders related to *Quatronicoccus* spp., as well as their tentatively affiliated catabolic gene lineages. We also 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  $^{13}\text{C}$ -labelled, suggesting that ecophysiological fine-tuning, rather than catabolic repertoire contributes to niche definition between aerobic and hypoxic degraders of BTEX compounds in groundwater systems.

## SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](#) online.

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