Anaerobic degradation of phenanthrene by a sulfate-reducing enrichment culture

Anne M. Himmelberg[1], Thomas Brüls[2,3], Zahra Farmani[4,5], Philip Weyrauch[4], Gabriele Barthel[1], Wolfgang Schrader[5], Rainer U. Meckenstock[4*]

[1]Institute of Groundwater Ecology, Helmholtz Zentrum München, Neuherberg, Germany

[2]CEA, DRF, Institut Jacob, Genoscope, Evry, France

[3]CNRS-UMR8030, Université Paris-Saclay, Evry, France

[4]Biofilm Centre, University of Duisburg-Essen, Essen, Germany

[5]Max-Planck-Institut für Kohlenforschung, Mülheim, Germany

*For correspondence. E-mail: rainer.meckenstock@uni-due.de; Tel. +49 (0)201 183-6601; Fax +49 (0)201 183-6603

Summary

Anaerobic degradation processes are very important to attenuate polycyclic aromatic hydrocarbons (PAHs) in saturated, anoxic sediments. However, PAHs are poorly degradable, leading to very slow microbial growth and thus resulting in only a few cultures that have been enriched and studied so far. Here, we report on a new phenanthrene-degrading, sulfate-reducing enrichment culture, TRIP1. Genome-resolved metagenomics and strain specific cell counting with FISH and flow cytometry indicated that the culture is dominated by a microorganism belonging to the *Desulfobacteraceae* family (60% of the community) and sharing 93% 16S rRNA sequence similarity to the naphthalene-degrading, sulfate-reducing strain NaphS2. The anaerobic degradation pathway was studied by metabolite analyses and revealed phenanthroic acid as the major intermediate consistent with carboxylation as the initial activation reaction. Further reduced metabolites were indicative of a stepwise reduction of the ring system. We were able to measure the presumed second enzyme reaction in the pathway, phenanthroate-CoA ligase, in crude cell extracts. The reaction was specific for 2-phenanthroic acid and did not transform other isomers.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/1462-2920.14335

The present study provides first insights into the anaerobic degradation pathways of three-ringed PAHs. The biochemical strategy follows principles known from anaerobic naphthalene degradation, including carboxylation and reduction of the aromatic ring system.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are among the most hazardous contaminants in the environment and pose a threat to all living organisms, as they are highly toxic and carcinogenic (Menzie *et al.*, 1992). Introduction of PAHs into the environment often occurs upon oil spills on sea and land, gas production and all downstream applications like automobile traffic or domestic heating (Johnsen *et al.*, 2005). Microbial degradation of PAHs, however, is negatively affected by the very low water solubility as well as by sorption to natural organic matter and sediments, decreasing the bioavailability

Aerobic biodegradation of PAHs is well understood (Horvath, 1972; Kiyohara and Nagao, 1978; Cerniglia, 1993; Juhasz and Naidu, 2000; Samanta *et al.*, 2002; Habe and Omori, 2003; Johnsen *et al.*, 2005). However, when large amounts of hydrocarbons get into saturated sediments, the habitat turns anoxic due to rapid oxygen consumption as co-substrate and electron acceptor. PAHs are then degraded anaerobically, which is an extremely slow process. So far, anaerobic degradation of PAHs has only been reproducibly demonstrated for naphthalene, methylnaphthalene, and phenanthrene (Zhang and Young, 1997; Annweiler, Richnow, *et al.*, 2000; Meckenstock *et al.*, 2000, 2016; Safinowski and Meckenstock, 2004; Davidova *et al.*, 2007).

Anaerobic degradation of naphthalene and methylnaphthalene has mostly been studied with the two sulfate-reducing cultures NaphS2 and N47 (Galushko *et al.*, 1999; Meckenstock *et al.*, 2000). Methylnaphthalene is activated at the methyl group by fumarate addition through naphthylmethylsuccinate synthase (Meckenstock *et al.*, 2004) and, after activation with CoA, degraded to the central intermediate 2-naphthoyl-CoA through beta-oxidation like reactions (Annweiler, Materna, *et al.*, 2000; Meckenstock *et al.*, 2000, 2016; Annweiler *et al.*, 2002). Naphthalene is activated through carboxylation (Zhang and Young, 1997; Meckenstock and

Mouttaki, 2011). After addition of CoA by 2-naphthoate-CoA ligase, the ring system is reduced in successive two electron reduction steps by the new type III aryl-CoA-reductases 2-naphthoyl-CoA reductase and 5,6-dihydro-2-naphthoyl-CoA reductase, yielding 5,6,7,8-tetrahydro2-naphthoyl-CoA (Eberlein, Estelmann, *et al.*, 2013; Boll *et al.*, 2014; Meckenstock *et al.*, 2016). The remaining aromatic ring I of the naphthalene skeleton is then reduced, most likely to hexahydronaphthoyl-CoA, followed by beta-oxidation like reactions and ring cleavage. The downstream degradation pathway proceeds via cyclohexane derivatives (Weyrauch *et al.*, 2017), and the central metabolism is reached via pimeloyl-CoA after the second ring cleavage (Weyrauch *et al.*, 2017).

So far, anaerobic phenanthrene degradation was reported for two sulfate-reducing cultures (Zhang and Young, 1997; Davidova *et al.*, 2007). Both cultures documented the production of phenanthroic acid which suggested that phenanthrene undergoes initial carboxylation similar to naphthalene or benzene (Kunapuli *et al.*, 2007; Dong *et al.*, 2017).

Here we report on a new anaerobic enrichment culture that is able to oxidize phenanthrene to CO_2 with sulfate as electron acceptor. The comparably fast-growing culture was characterized and indications on the anaerobic phenanthrene degradation pathway are presented based on metabolite and enzyme analyses.

Results

Enrichment of culture TRIP1

The enrichment culture TRIP1 was grown in batch cultures with phenanthrene as sole carbon and electron sources and sulfate as electron acceptor. The generation time was approximately 10 days and the culture needed around three months before the stationary phase was reached (Figure 1). The stoichiometric equation for sulfate reduction to sulfide with phenanthrene as electron donor is $C_{14}H_{10} + 8.25\ SO_4^{2-} + 9\ H_2O \rightarrow 14\ HCO_3^- + 8.25\ HS^- + 5.75\ H^+$, yielding a Gibbs free energy of -341 kJ with a possible energy conservation of ca. 5 ATP per mole of phenanthrene oxidized to CO_2 (McFarland and Sims, 1991; Meckenstock *et al.*, 2016). In culture TRIP1, sulfate depletion of 2.42 mM was coupled to phenanthrene depletion of 0.08 mM (theoretical concentration in the

aqueous phase, as calculated from the phenanthrene loss in the HMN carrier phase). The resulting electron recovery of 341% can be explained with the loss of phenanthrene by absorption into the butyl stopper. Nevertheless, we propose that culture TRIP1 fully oxidizes phenanthrene to CO_2, as members of the *Desulfobacteraceae* are commonly documented as complete oxidizers.

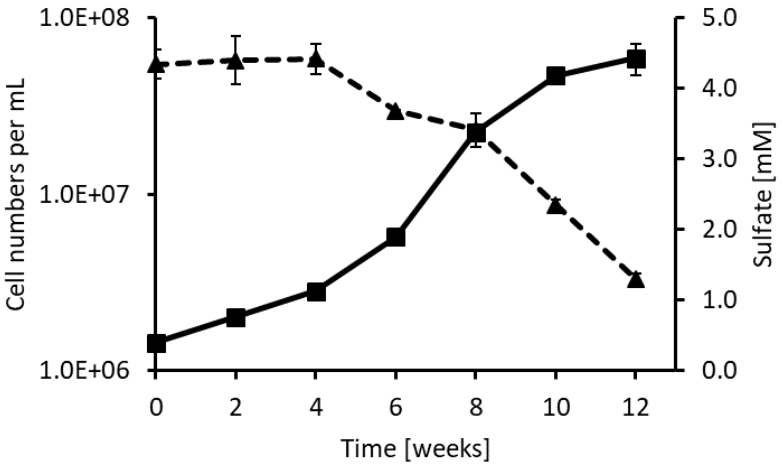


Figure 1: Growth of culture TRIP1 with phenanthrene as sole carbon and electron source and sulfate as electron acceptor. Solid squares: cell numbers per mL of culture. Solid triangles: sulfate concentrations, determined by the barium chloride method (Tabatabai, 1974). Error bars depict standard deviations of triplicate incubations.

The growth curve of culture TRIP1 showed an initial lag phase of about four weeks. Even a fully-grown culture never turned turbid, but the very little biomass accumulated at the bottom of the serum vial.

Growth tests were conducted with sulfate, nitrate, elemental sulphur, or iron(III) (ferrihydrite) and without a terminal electron acceptor. Culture TRIP1 was neither able to utilize nitrate nor ferrihydrite as electron acceptor with phenanthrene as electron donor and did not grow without a terminal electron acceptor. When elemental sulfur was added as electron acceptor, the culture performed sulphur disproportionation, thus producing significant amounts of sulfide (7 mM) and

sulfate (3.5 mM), as determined by ion chromatography. However, terminal restriction fragment length polymorphism analysis (T-RFLP) of PCR products derived from 16S rRNA genes revealed shifts in the culture composition during sulfur disproportionation. We therefore conclude that sulfur disproportionation was not performed by the dominating phenanthrene-degrading microorganism.

Batch incubations were conducted with different electron donors and sulfate as electron acceptor to elucidate the substrate spectrum of the culture. When growth was detected in the second transfer by significantly increasing cell counts (ten times higher than the control without substrate, cell counts conducted with flow cytometry), T-RFLP analysis was performed to assess whether the microbial community remained constant, and the same organisms were responsible for the degradation activity. Culture TRIP1 was able to grow with the positive control phenanthrene as substrate but not with naphthalene, anthracene, acenaphthene, or fluoranthene. The dominant peaks in the T-RFLP analysis of the cultures grown with phenanthrene were at 53 bp and 74 bp. The culture showed growth with the carboxylated aromatic compounds 2- and 4-phenanthroic acid, 2-naphthoic acid and terephthalic acid. However, the dominant T-RFLP peaks at 53 bp and 74 bp were only apparent with 2-phenanthroic acid and 4-phenanthroic acid as substrates, consistent with the same organisms transforming these substrates. With terephthalic acid a new T-RFLP peak (at 156 bp) became dominant, suggesting that this compound is not relevant to the phenanthrene-degrading organisms. A similar pattern was observed for benzoic acid and benzene as substrates (T-RFLP peak at 515 bp).

Substrate	Total cell density[a] [cells/ml]	T-RFLP fragment length bp [b]	Assignment to organism
Phenanthrene (positive control)	6.8 x 10^6 (+)	**53**; 74	*Desulfatiglans*-1
Naphthalene	*(*)*	–	
Acenaphthene	*(*)*	–	
Anthracene	*(*)*	–	
Fluoranthene	*(*)*	–	

Benzene	7.0 x 10^6 (+)	53; 74; **515**	*Desulfatiglans*-2
Benzoic acid	5.1 x 10^7 (+)	53; 74; **515**	*Desulfatiglans*-2
1-Naphthoic acid	2.3 x 10^6 (+)	*(*)*	
2-Naphthoic acid	2.7 x 10^6 (+)	**53**; 74; 97	*Desulfatiglans*-1, unclassified bacterium
1,2,3,4-Tetrahydro-2-naphthoic acid	5.3 x 10^4 (-)		
5,6,7,8-Tetrahydro-2-naphthoic acid	5.2 x 10^4 (-)		
2-Phenanthroic acid	1.3 x 10^7 (+)	53; **404**	unknown
4-Phenanthroic acid	7.1 x 10^5 (+)	**53**; 74	*Desulfatiglans*-1
Terephthalic acid	8.6 x 10^6 (+)	**156**	unknown

Table 1: Substrate utilization by culture TRIP1. a) Growth was evaluated as positive (+) if the cell count in the second transfer increased more than ten-fold compared to the control without terminal electron acceptor (9.8 x 10^4), as measured by flow cytometry, (-) bacterial growth lower than ten-fold compared to negative control or no detectable growth, (*) PCR amplification not possible. b) Fragment length in bold describes the dominant peaks in the TRFLP analysis.

Microbial community composition of the TRIP1 culture

Genome-centric metagenomic analyses (Gkanogiannis *et al.*, 2016) of the community genome from the culture TRIP1 led to the reconstruction of a handful of nearly complete genomes, including the genome of the most prominent organism. Table S1 (Supplemental Material) displays the ten organisms from NCBI's Refseq database (https://www.ncbi.nlm.nih.gov/refseq) that are more closely related to the dominant *Desulfatiglans* organism in terms of genome-level syntenic relationships (Vallenet *et al.*, 2006, 2013). The 16S rRNA gene sequence of this bacterium was most closely related to *Desulfatiglans anilini* strain Ani1 (= DSM 4660(T)) and *Desulfatiglans anilini* strain AK1 (94% identity) -the latter were recently reclassified as *Desulfatiglans anilini* species

(Suzuki *et al.*, 2014) and *Desulfatiglans* spec. strain NaphS2 (93% similarity, Galushko *et al.*, 1999), with only weaker (87%) similarity to the Phe4A strain from the phenanthrene-degrading culture Phe4 (Davidova *et al.*, 2007). Other organisms with nearly complete reconstructed genomes showed similarities to *Desulfatiglans anilini* strain AK1 (98% similarity), *Paludibacter propionicigenes* (91% similarity) as well as to an unknown *Spirochaetales*. As the similarity to the *Spirochaetales* is deduced from genome-level syntenies but no 16S rRNA sequence could be recovered from the corresponding organism in the TRIP1 culture, it is not included in the tree depicted in Figure 2.

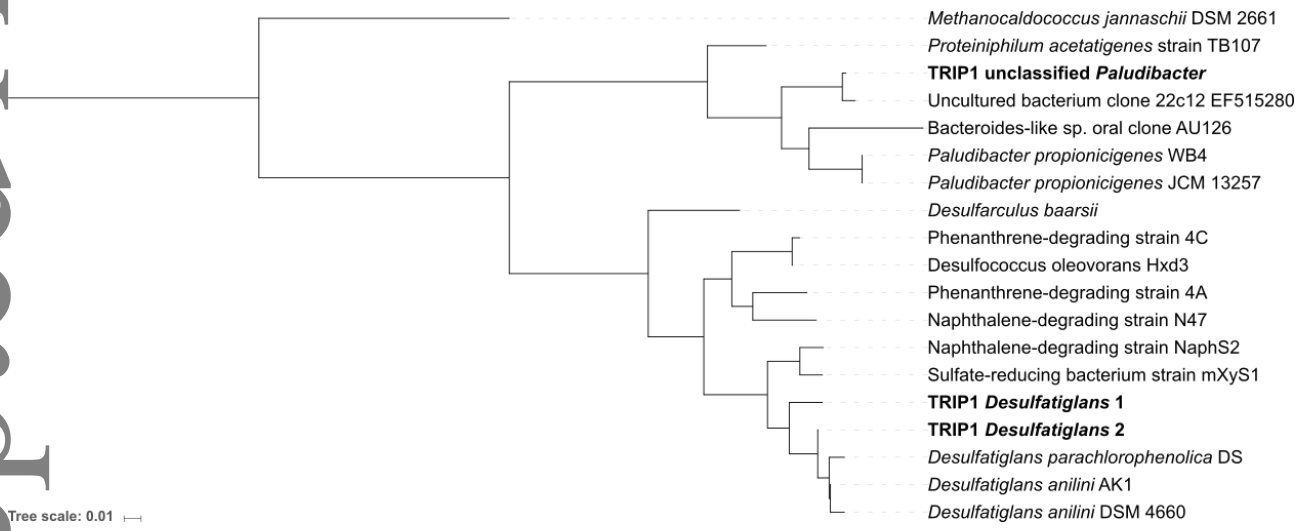


Figure 2: Phylogenetic tree of selected members of the enrichment culture TRIP1, including the dominant *Desulfobacteraceae organism (Desulfatiglans-1)*, and closely related species together with other known PAH-degrading bacteria, based on neighbor-joining analysis of full length 16S rRNA gene sequences obtained from the metagenome. Evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.*, 2011) and are expressed in units of base substitutions per site. Members of the TRIP1 enrichment culture are marked in bold. *Geobacter* and *Paludibacter* like species were present at less than 2% relative abundance, and were not included in this analysis.

The completion and contamination levels of the reconstructed genomes (Table 2) were evaluated using lineage-specific single copy gene markers and the CheckM software (Parks *et al.*, 2015). With this method we were able to generate two almost fully closed genomes of the main dominating bacterium and the bacterium that is closest related to a *Geobacter* species.

Organism	Marker lineage	# genomes	# markers	# uniquely found markers	Completeness	Contaminatio
Dominant *Desulfobacteraceae* organism	Deltaproteobacteria (UID3216)	83	247	244	99.35	0.97
Geobacter-like organism	Bacteria (UID2495)	2993	147	143	99.94	5.49
Paludibacter-like organism	Bacteria (UID2569)	434	278	266	95.61	0.27
Spirochaete	Bacteria (UID2495)	2993	141	140	98.85	0.00

Table 2: Genome completion estimates of metagenomic "bins" constructed from culture TRIP1. Strain heterogeneity values (not shown in the Table) were 0.00 for all the genomes.

Relative abundance estimates of the organisms with nearly complete genomes were obtained by mapping the original shotgun reads using the BWA software (Li and Durbin, 2009), leading to a 60.4% figure for the first unclassified *Desulfobacteraceae* (*Desulfatiglans*-1), 2.1% for the second Desulfobacteraceae (*Desulfatiglans*-2), 1.2% for the unclassified *Bacteroidetes* and 0.51% for the unknown *Spirochaete* bacterium. The estimate for the dominant *Desulfobacteraceae* (*Desulfatiglans*-1) could be independently confirmed by flow cytometric counting of samples from the TRIP1 culture stained with fluorescence in situ hybridisation (FISH) probes specific for this organism. Flow cytometry analysis of the community stained with these FISH probes revealed that about 50% of the cells belonged to the dominating *Desulfatiglans* species with 3.4×10^6 counts (TRIP_Desulfo183-Fam, single measurement due to low amount of sample) out of a total cell count of 6.8×10^6 cells (sd = 4.8×10^6).

The cell counts in the different terminal electron acceptor assays that had no visible growth were two orders of magnitude lower than in the phenanthrene control culture with sulfate as electron

acceptor. The cell densities for the cultures growing with 2-phenanthroic acid and benzoic acid as electron source were ten-fold higher than the culture growing with phenanthrene. After 12 weeks of incubation with phenanthrene and sulfate, the culture was subjected to metabolite analysis using GC-MS. The results show that a set of different carboxylated and partly hydrogenated phenanthrene derivatives were detected.

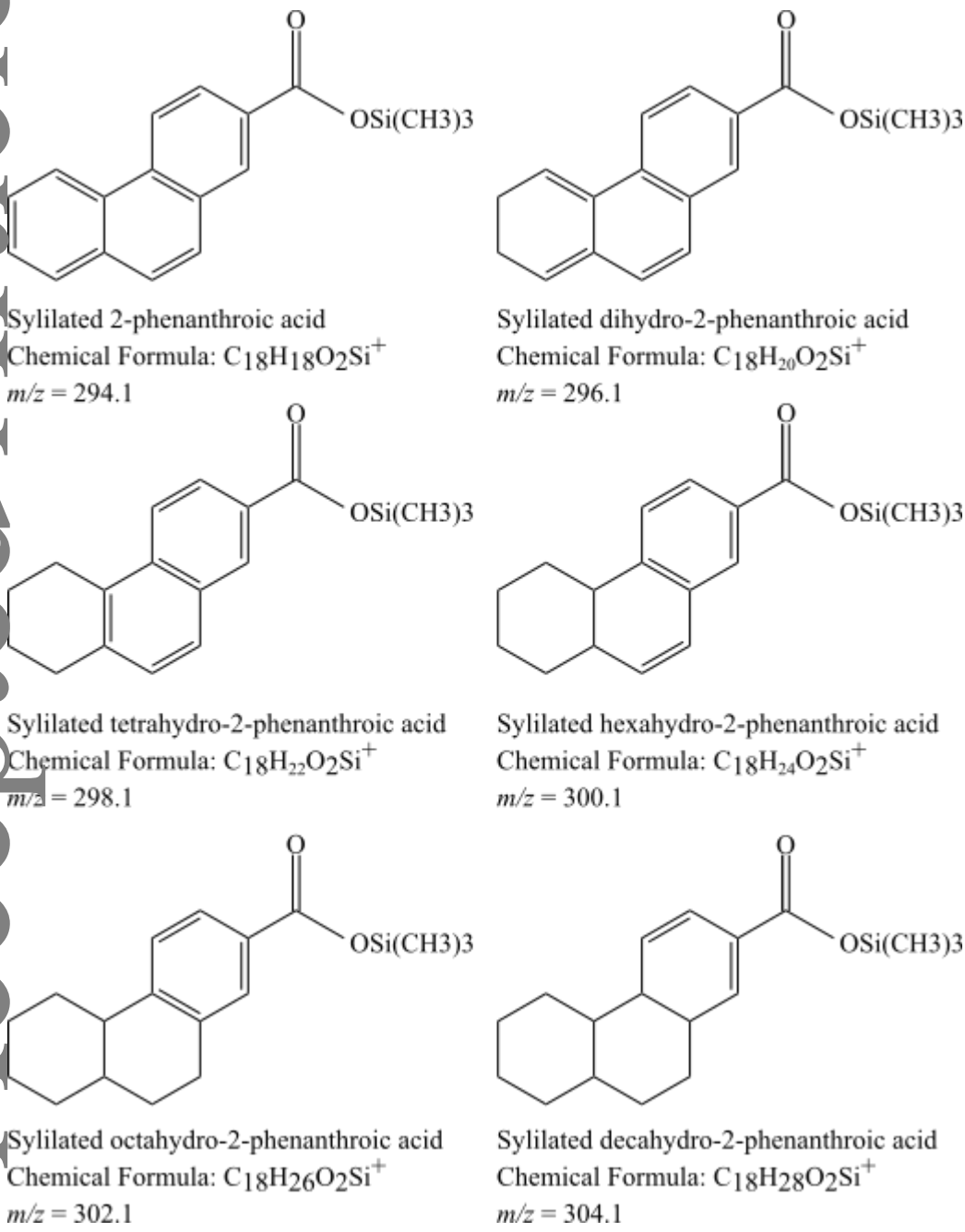


Figure3: Molecular masses and possible structures of metabolites characterized in culture TRIP1 by GC-MS; metabolites are depicted as ions of sylilated acids.

In order to confirm the structure of the metabolites shown in Figure 3, *collision induced*

dissociation (CID) was performed for the target *m/z* range of 221.06 (phenanthroic acid) in ESI negative mode. Figure 4 shows a part of the high resolution MS spectrum of phenanthroic acid after CID, which resulted in the loss of a water molecule and the carboxyl group.

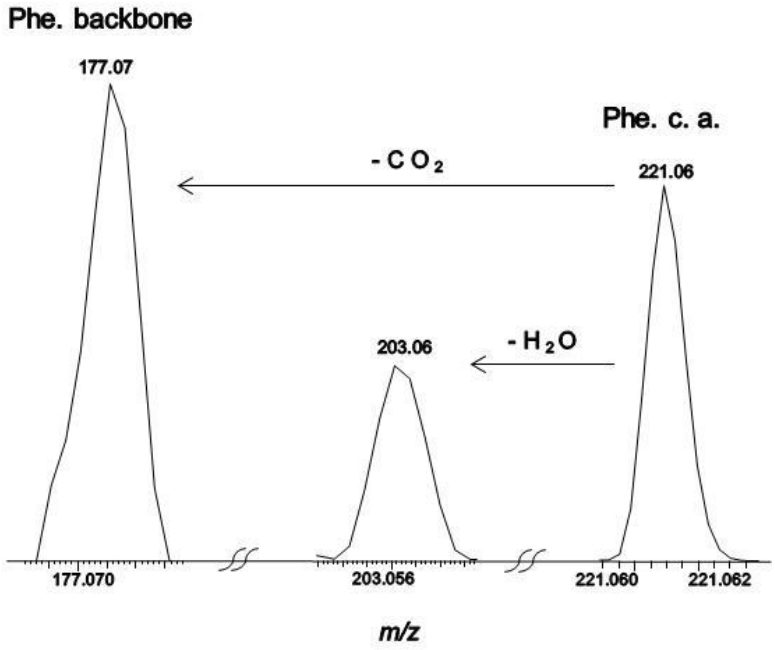
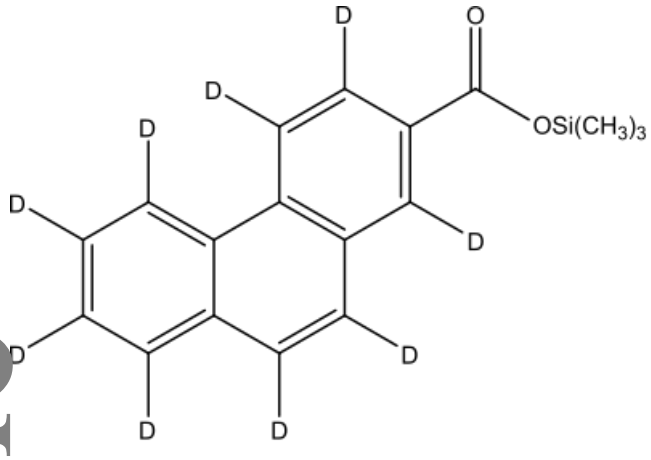
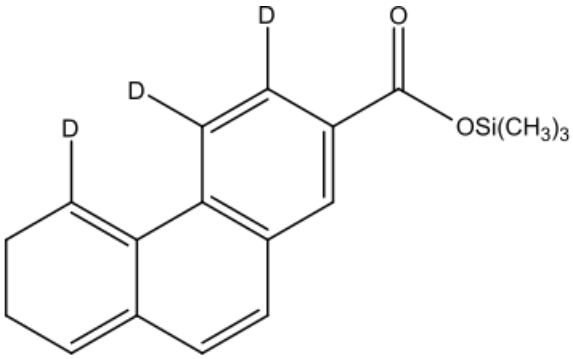


Figure 4: High resolution-MS spectrum of the phenanthroic acid (Phe. c. a.) in negative mode, showing loss of H_2O and CO_2 through CID from *m/z* 221.0.

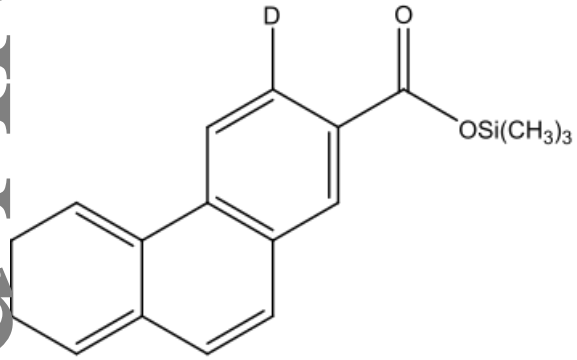
The detected metabolites indicate a stepwise ring reduction of 2-phenanthroyl-CoA. Thus, we assume a ring reduction starting from ring III of phenanthroic acid, opposite to the carboxyl group, similar to the anaerobic reduction of naphthalene (Eberlein, Johannes, *et al.*, 2013; Estelmann *et al.*, 2015). For further elucidation of the degradation pathway, culture TRIP1 was grown with fully deuterated phenanthrene ($C_{14}D_{10}$) as substrate. Metabolites were extracted and analyzed with GC-MS. A number of putative metabolites of phenanthrene degradation containing deuterium were detected, indicating that they originated from the deuterated phenanthrene. The measured molecular masses supported the metabolite analysis with non-labeled phenanthrene indicating a stepwise reduction of phenanthroyl-CoA. However, the calculated number of saturated and unsaturated bonds did not agree with the number of deuterium atoms in the molecules, indicating that isotope exchange with protons from water occurred during the ring reduction process (Figure 5).



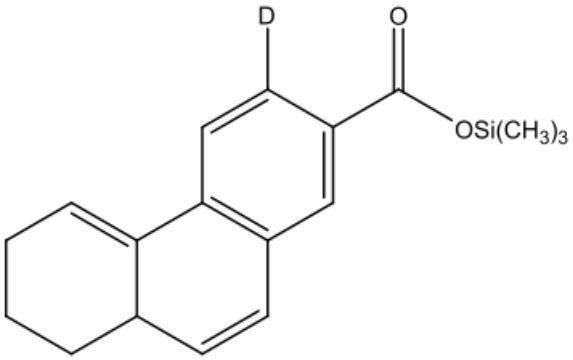
Silylated 2-phenanthroic acid (D_{10})
m/z = 303.16



Silylated 6,7-dihydro-2-phenanthroic acid (D_3)
m/z = 299.14



Silylated 6,7-dihydro-2-phenanthroic acid (D_1)
m/z = 297.13



Silylated 6,7,8,9-tetrahydro-2-phenanthroic acid (D_1)
m/z = 299.14

Figure 5: Characterized metabolites of culture TRIP1, incubated with fully deuterated phenanthrene, measured by GC-MS (metabolites are depicted as ions of silylated acids).

Detection of enzymes involved in the anaerobic phenanthrene degradation

The first metabolite in anaerobic phenanthrene degradation is assumed to be phenanthroic acid (Zhang and Young, 1997; Davidova *et al.*, 2007). In order to identify which isomer of phenanthroic acid was produced by the carboxylation reaction, we compared the LC-MS retention time of the phenanthroic acid metabolite found in culture supernatants, with the LC-MS chromatograms of three reference phenanthroic acid isomers, i.e. 2-phenanthroic acid, 4-phenanthroic acid, and 9-phenanthroic acid. The two isomers 1-phenthroic acid and 3-phenthroic acid were not commercially available.

We then measured the phenanthroate-CoA ligase reaction, the presumed second reaction in the anaerobic phenanthrene degradation, in crude cell extracts. The reaction was started with the addition of substrate to the reaction mixture. The reaction with 2-phenanthroic acid was very fast, so that the first sample immediately taken after the start of the reaction already contained CoA-esters (Figure 6). After 10 min, all 2-phenanthroic acid was converted to phenanthroyl-CoA. The phenanthroate-CoA ligase reaction was not observed with 4, or 9-phenanthroic as substrate, indicating that its natural substrate is 2-phenanthroic acid and further indicating that the latter is the product of phenanthrene carboxylase.

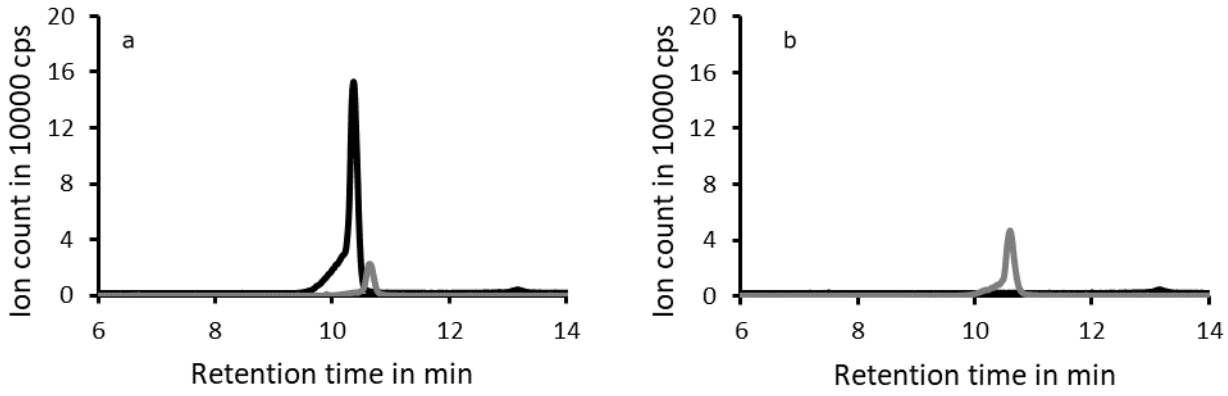


Figure 6: Enzyme assay of the phenanthroate-CoA ligase reaction with cell extracts of culture TRIP1 with 2-phenanthroic acid (black line, *m/z* = 221 (-)) as substrate. The production of 2-phenanthroyl-CoA was followed over time (grey line, *m/z* = 972(+)). Panel a) t = 0 min, Panel b) t = 10 min.

Comparing the retention times and mass spectra of the phenanthroyl-CoA produced in the cell extracts of culture TRIP1 with the synthesized CoA-esters of the three reference phenanthroic acids (i.e. 2-, 4-, and 9-phenanthroic acid) provided additional support that the natural product is 2-phenanthroyl-CoA (Figure 7).

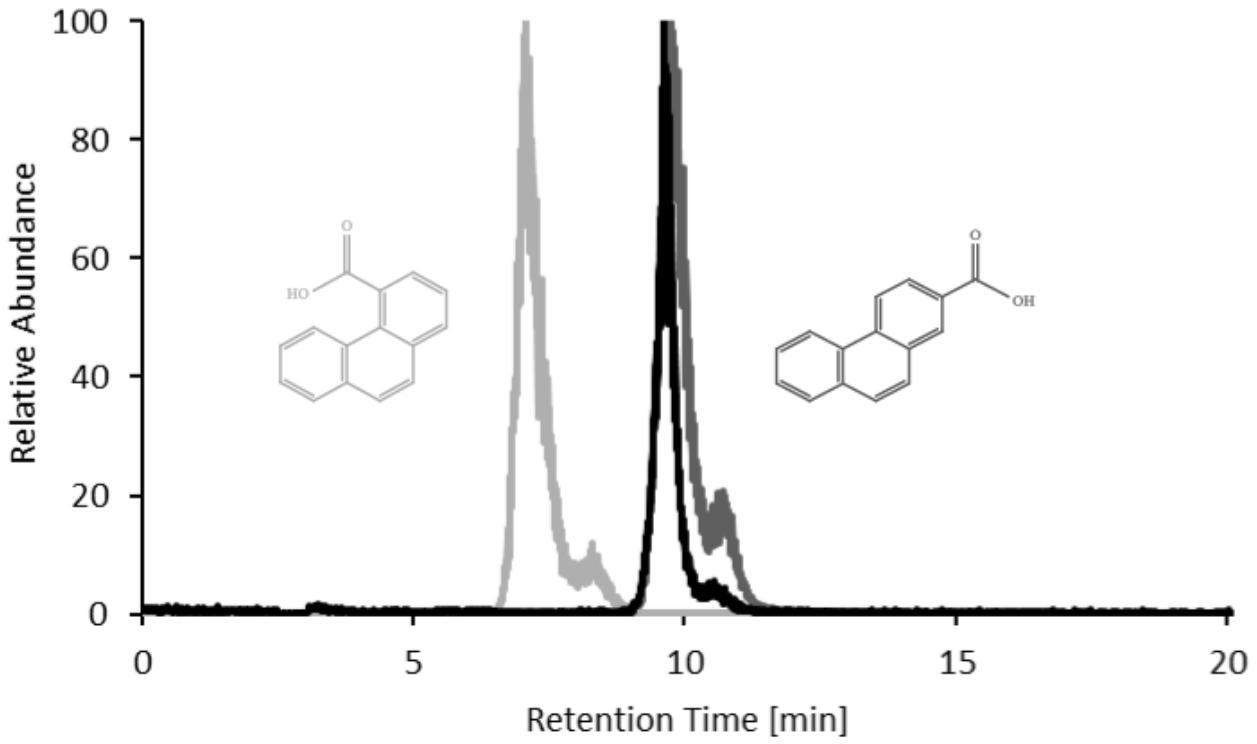


Figure 7: LC-MS chromatograms of 2- (dark grey) and 4-phenanthroic acid (light grey) compared to extracted phenanthroic acid from phenanthroate-CoA ligase enzyme assays performed with crude cell extracts of culture TRIP1 (black).

However, as 1- and 3-phenanthroic acid are not commercially available, we could not test them as substrate and hence cannot exclude that they might be converted by the ligase. Other enzyme reactions such as phenanthrene carboxylase and phenanthroyl-CoA reductase were also tested but could not be directly measured.

Discussion

The investigation of anaerobic degradation of polycyclic aromatic hydrocarbons (PAHs) is still in its infancy (Meckenstock *et al.*, 2016). Among three or more ringed PAHs, only phenanthrene has been reported to be reproducibly degraded under strict absence of molecular oxygen. Following the detection of phenanthroic acid in culture supernatants of two independent enrichment cultures, carboxylation was proposed as the initial activation step in anaerobic phenanthrene degradation (Zhang and Young, 1997; Davidova *et al.*, 2007). Furthermore, ^{13}C-labelled carbonate was shown to

be incorporated into the carboxyl group of phenanthroic acid (Zhang and Young, 1997; Davidova *et al.*, 2007), providing further support for a carboxylation reaction. This reaction is analogous to anaerobic naphthalene degradation where the initial enzymatic carboxylation reaction was measured in dense cell suspensions (Mouttaki *et al.*, 2012).

However, more comprehensive information on the anaerobic degradation of phenanthrene is still lacking. In order to investigate anaerobic phenanthrene degradation in more detail, we enriched an anaerobic phenanthrene-degrading culture with sulfate as electron acceptor with an inoculum from the Pitch Lake in Trinidad and Tobago. The culture degraded phenanthrene with rates similar to anaerobic naphthalene-degrading cultures (6-8 days doubling times for NaphS2, and more than two weeks for N47, Galushko *et al.*, 1999; Meckenstock *et al.*, 2000), which is remarkable given the much lower aqueous solubility of phenanthrene (7.3 µM at 30° C for phenanthrene versus 242 µM at 25°C for naphthalene). This relatively fast growth allowed studying the culture in more detail.

The dominant organism accounted for more than 60 % of all microbial cells in the culture and its 16S rRNA gene sequence shared 94% and 93% identity to *Desulfatiglans anilini* strain Ani1 and the naphthalene-degrading strain NaphS2, respectively. Assuming that this organism is responsible for phenanthrene degradation, it appears only remotely related to the previously reported phenanthrene-degrading microorganism Phe4A (Davidova *et al.*, 2007). Its 16S rRNA gene sequence affiliates to the family *Desulfobacteriaceae*, and more specifically to the genus *Desulfatiglans,* which already includes several sulfate-reducing members that are known to degrade aromatic hydrocarbons, such as the aniline-degrading *Desulfatiglans anilini (*former *Desulfobacterium anilini)*, or the naphthalene-degrading strains NaphS2 and N47 (Schnell *et al.*, 1989; Galushko *et al.*, 1999; Meckenstock *et al.*, 2000).

Other members in culture TRIP1 include Spirochaetes, members of which (e.g. Spirochaetaceae*)* are often found in anaerobic cultures capable of degrading aromatic compounds and have been identified in sediment, ground- and freshwater samples. Their role of these Spirochaetes appears related to necromass fermentation, leading to a microbial loop recycling nutrients (Koelschbach *et*

al., 2017; Dong *et al.*, 2018).

The reconstructed genome sequences are currently analysed and will be reported elsewhere, but as the enzymes for the anaerobic degradation of phenanthrene are currently unknown, genomic sequences by themselves do not point to obvious candidate genes for the degradative enzymes. For example, putative genes encoding naphthoyl-CoA ligase from the TRIP1's dominant organism share only 35% identity to the closest related sequence from culture NaphS2. On the other hand, some genes encoding flavoenzymes of the "old yellow enzyme" (OYE) family, a large family that includes naphthoyl-CoA reductases (Eberlein, Estelmann, *et al.*, 2013), showed stronger similarities (up to 60% identity) to NaphS2 OYEs, and are plausible candidates for encoding similar activities on phenanthrene-derived substrates. These limitations prompted us to derive information on the degradation pathway from metabolite analysis of culture supernatants. The dominant metabolite was phenanthroic acid, consistent with observations from the other two known phenanthrene-degrading cultures (Zhang and Young, 1997; Davidova *et al.*, 2007).

Phenanthroic acid was convincingly measured as a real metabolite of the degradation pathway in phenanthroate-CoA ligase enzyme assays performed on crude cell extracts of culture TRIP1. Among the three tested isomers (2-, 4-, and 9-phenanthroic acid), only 2-phenanthroic acid was converted, indicating that the measured ligase reaction is specific and not due to a non-specific side reaction. The measurement of this enzymatic activity in anaerobic phenanthrene degradation also indicates that the initial carboxylation reaction most likely produces 2-phenanthroic acid, which is similar to anaerobic naphthalene degradation where 2-naphthoic acid is the product of the naphthalene carboxylase reaction (Mouttaki *et al.*, 2012). However, due to the lack of the 1- and 3-phenanthroic acid isomers as reference compounds or substrates, it cannot be excluded that these isomers can also be converted by phenanthroate-CoA ligase.

GC-MS analysis of the TRIP1 culture revealed further derivatives of phenanthroic acid with molecular masses indicative of a stepwise reduction of phenanthroyl-CoA, which is again analogous to the biochemical strategy in anaerobic naphthalene degradation, where the ring system

is reduced in two-electron steps to overcome the resonance energy and prepare the compound for further degradation (Annweiler *et al.*, 2002; Eberlein, Estelmann, *et al.*, 2013). This model was further supported by analyzing metabolites arising from the degradation of deuterated phenanthrene, among which reduced compounds were characterized. However, the seemingly erratic conservation of deuterium atoms in the metabolites of deuterated phenanthrene degradation suggests that some detected metabolites might undergo isotope exchange reactions with protons, either in the culture or later during the chemical analysis. Therefore, it cannot be excluded that some of the detected reduced phenanthroic acid derivatives do not arise from the degradation pathway, even though a stepwise reduction of the aromatic ring system is highly probable.

Our study sheds light on the first steps of the anaerobic degradation pathway of phenanthrene, and indicates that the biochemical strategies identified in the anaerobic degradation of naphthalene and benzene (Meckenstock *et al.*, 2016) also hold for larger non-substituted three-ringed PAHs, namely a carboxylation as the initial activation reaction and a stepwise reduction of the aromatic ring system to overcome the resonance energy. The biochemical evidence provided here opens the way to the identification of further degradation reactions and the corresponding genes.

Experimental Procedures

Origin of the culture and growth conditions

The phenanthrene-degrading culture TRIP1 was enriched from 5 mL inoculum of a muddy soil-oil mixture at the Pitch Lake in La Brea, Trinidad. It was cultivated in 100 mL serum bottles with 50 mL of anoxic freshwater mineral medium as described previously (Widdel *et al.*, 1983; Widdel and Bak, 1992; Meckenstock *et al.*, 2000) with 20 mM sulfate as terminal electron acceptor. Phenanthrene was dissolved (1.5%) in 2,2,4,4,6,8,8-heptamethylnonane (HMN) (20 mL/L culture volume) and added to the medium as an overlay. The serum bottles were closed with butyl stoppers (Glasgerätebau Ochs, Göttingen, Germany) and the headspace was exchanged with N_2:CO_2 (80:20). Bottles were incubated at 30°C in the dark. After growth became visible as a faint turbidity, the

enrichment was transferred (10%, v/v) every three months. Media preparation and all handling procedures of the culture were strictly anoxic. 1.6 L cultures were cultivated accordingly in 2 L Schott bottles (Schott AG, Mainz, Germany) for three months.

Substrate Tests

For substrate tests, compounds were dissolved in HMN (1.5%), i.e. phenanthrene, naphthalene, benzene. 2- and 4-phenanthroic acid were added directly with a molar equivalent of NaOH to a final concentration of 500 µM. 1- and 2-naphthoic acid, 1,2,3,4-tetrahydronaphthoic acid, 5,6,7,8-tetrahydronaphthoic acid, terephthalic acid, benzoic acid were added from 5 mM aqueous stock solutions with a molar equivalent of NaOH to a final concentration of 1 mM in the culture. Acenaphthene, acenaphthylene, anthracene, and fluoranthene were dissolved in acetone, the necessary amount for a final concentration of 1 mM distributed evenly in the serum bottles and dried overnight in the fume hood before adding the medium.

Electron-acceptor tests

As alternative electron acceptors were added: elemental sulphur (spatula), nitrate (5 mM), and ferrihydrite (80 mM).

Determination of cell counts

Samples were taken with a syringe through the stopper. The collected samples (0.4 mL) were mixed with 0.4 mL of water and 200 µL of 4,6-diamidino-2-phenylindole (DAPI) (5 µg/mL end concentration). The mixture was incubated for 20 min in the dark, filtered over sterile black Isopore membrane filters (0.2 µm, GTBP, Merck Millipore, Ireland) and subjected to fluorescence microscopy (Axio Scope A1 microscope. Zeiss, Germany).

Fluorescence in situ hybridization (FISH) analysis

Based on the 16S rRNA gene sequences derived from metagenomic shotgun sequencing and using the ARB software package (Ludwig *et al.*, 2004), a specific FISH probe TRIP_Desulfo183-Fam (GACCAAAGUCUCUUGGAC) was designed for the dominant *Desulfatiglans* in the culture as well as another probe with specificity for the Desulfobacteraceae (TRIP-Desulfo1430-Cy3, GUUAGCCCAGCACCUUCU) family. Samples were fixed by adding 4% paraformaldehyde to a final concentration of 1% and stored at 4°C until use. For microscopic analysis, 20 µL aliquots were dried for 90 min at 46°C on a microscopic slide and dehydrated by successively increasing ethanol concentrations of 50, 80 and 99% for 3 min each. Hybridization was performed for 2.5 h, followed by a 30 min washing step according to a previously published protocol (Pernthaler *et al.*, 2001). Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and embedded in Citiflour (Electron Microscopy Sciences, Hatfield, USA).

TCC analysis, cell counting with FISH Probe

For flow cytometric cell counting, 10 µL of fixed cell mixture were dried in an oven at 46°Celsius for one hour. The dried cells were washed with 99% ethanol, centrifuged at 4000 × g for 2 min at room temperature, and the ethanol decanted. The pellet was dried at 46°C for 10 min. 100 µL of hybridization buffer (see Pernthaler *et al.*, 2001) and 5 µL of FISH probe TRIP_Desulfo183-Fam were added to the mixture and incubated for two hours at 46°C. After a second centrifugation step, the washing buffer (Pernthaler *et al.*, 2001) was added and incubated at 46°C for 30 minutes. After another centrifugation step, the washing buffer was completely removed and the cell pellet resuspended in 100 µL PTN buffer (120 mM Na_2HPO_4, 125 mM Tris, 0.25 mM NaCl, pH 8). Stained cells were injected to a Flow Cytometer (FC500 Cytomics; Beckman Coulter). The detector was set to 398 for the forward-angle light scatter, 972 for the side-angle light scatter, the channel for green and red fluorescence were at 569 and 640 volts, respectively. Measuring time for all samples was 60 seconds and the maximum events were set to 10 million at a medium flow rate. Cell counts were calibrated using Trucount BD Beads (Trucount™ Absolute Counting Tubes, BD Bioscience,

Franklin Lakes,NJ).

Sulfate determination with barium gelatin

Sulfate concentrations were determined with the barium chloride method (Tabatabai, 1974). The barium gelatin reagent was prepared by dissolving 0.75 g of gelatin from bovine skin (Sigma-Aldrich) in 250 mL of boiling purified water. After cooling in an ice bath 10 g of barium chloride were added.

The barium gelatin reagent was mixed 1:1 with 0.5 N HCl and 10% volume of sample was added. Samples were measured in biological and technical triplicates in a plate reader (Wallac Victor3 1420; PerkinElmer, Waltham, MA) at 450 nm after agitation for 10 minutes.

Sulfate measurement with ion chromatography

Samples (150 µL) were centrifuged at 13,000 × *g* for 10 min to remove solids. 100 µL of the supernatant were diluted with 900 µL, 0.1 M KOH to precipitate salts, incubated for 10 minutes and subjected to a second centrifugation step. 20 µL of the sample were mixed with 980 µL of purified water and measured on an Aquion IC system (Thermo Fisher Scientific GmbH, USA).

Sulfide determination

Sulfide concentrations were measured as described by Fischer (1883) and Cline (1969).

Quantification of phenanthrene depletion

For quantification of the phenanthrene depletion in the HMN carrier phase, a set of 150 mL cultures (one control sample and three biological samples) was grown for 12 weeks. The culture medium was separated in a separation funnel and the resulting HMN phase was dried over magnesium sulfate ($MgSO_4$). The magnesium sulfate was removed by centrifugation at 240 x g for 5 minutes. The supernatant was mixed with an equal volume of 12 mg/ ml phenanthrene D_{10} standard and

diluted 1:500 (v/v) with toluene for quantification by APPI-triple quad-MS.

Metabolite extraction for GC-MS analysis

150 mL sample were taken from a 1.5 L culture and the HMN phase was removed in a separating funnel. The remaining water phase was first brought to a pH of 12 to lyse all cells and cleave ester bonds with 10 M NaOH, and after 15 minutes of stirring acidified with 6 M hydrochloric acid to pH 2. Carboxylic acids and aromatic alcohols were extracted three times with 50 mL ethyl acetate. The ethyl acetate phase was dried with pulverized sodium sulfate dried by vacuum evaporation and resolubilized with 500 µL ethyl acetate. Resolubilized extracts were either derivatized once with bis(trimethylsilyl) trifluoroacetamide (BSTFA, Sigma-Aldrich, St. Louis, USA) for 30 minutes at 60°C, or with trimethylsulfonium hydroxide (TMSH, Sigma-Aldrich, St. Louis, USA). Derivatized samples were analyzed with GC-MS.

LC-MS analysis

LC-MS analyses (liquid-chromatography coupled to mass-spectrometry) were performed with a LC-2040C system coupled to a LC-MS-2020 single quadrupole mass-spectrometer (Shimadzu Deutschland, Duisburg, Germany). Samples were separated via a Nucleodur C_{18} Pyramid column, 100 × 3 mm, 5 µm particle size (Macherey-Nagel, Dueren, Germany). The column oven was set to 35 °C. Eluent A was water with 0.1% (w/v) ammonium formate, eluent B acetonitrile. Eluent B increased from 10% up to 40% over 15 min at a flow rate of 0.7 mL/min. Mass spectrometric analysis was carried out with an ESI system in positive mode. The voltage of the ESI system was set to 4.5 kV, the temperature to 350 °C. Nebulising gas flow was 1.5 L/min, drying gas flow 12 L/min. Heat block temperature was 200 °C and the desolvation line was operated at 0 V and 250 °C. Mass-to-charge ratios (*m/z*) of expected metabolites were detected in single ion mode with ionisation conditions as optimised for 2-naphthoyl-CoA. Settings for the Qarray™ ion guide were as follows: DC voltage 0 V, RF voltage 120 V.

A chemical standard of 2-phenanthroyl-CoA was synthesised in a small-scale reaction following a two-step procedure (Kawaguchi *et al.*, 1981; Peter *et al.*, 2016). First, 2-phenanthroic acid was activated with carbonyldiimidazole (CDI) in tetrahydrofuran (THF). In a second step, the CDI-activated intermediate was directly converted into the CoA-thioester by addition of free CoA-SH in THF/water buffered with 100 mM $NaHCO_3$. The product could be used as a standard for LC-MS analysis without further purification.

Gas chromatography – Electron Ionization-Mass Spectometry

All silylated samples were analyzed using an ISQ™ LT Single Quadrupol GC-MS-System (Thermo Fisher Scientific, Germany). Ionization was performed by EI at 70 eV.

For GC separation, 3 µL of derivatized sample were injected splitless to the GC system equipped with a Rxi-5 MS 8 capillary column (30 m x 0.25 mm x 0.25 µm). The injector was set to 240°C. The carrier gas was helium with a flow rate of 1.5 mL/min. The operating temperature of the injector was 5 min at 80°C, ramp with 4°C/min to 280°C, 5 min constant at 280°C, ramp with 10°C/min to 330°C, 5 min constant at 330°C. The ionization temperature was maintained at 300°C. Electron ionization (EI) with 70 eV in positive mode was used as ionization technique. The mass spectra were interpreted using MassLib software (MassLib™ V9.4 Release, MSP Kofel, Switzerland) by matching to the NIST and also the local MPI KoFo (Max-Planck-Institut für Kohleforschung, Mülheim an der Ruhr, Germany) databases for assignment of observed metabolites. Isomeric characterization of the detected phenanthrene carboxylic acid was performed through comparison of the retention times of commercially available standards (2- and 4-phenanthroic acid).

CID (collision induced dissociation) – ESI-LTQ Orbitrap Mass Spectrometry

The mass spectrometric analysis of the extracted metabolites was performed using a research grade Orbitrap Elite (Thermo Fisher Scientific, Germany) with mass resolution set to 480,000 (FWHM at

m/z 400). Negative mode ESI was used for ionization with the following conditions: ionization potential of 4.2 kV, sheath gas flow of 5.0, auxiliary gas flow of 2 and sweep gas flow rate of 0 (arbitrary units). The transfer capillary temperature was set at 275 °C. The identity of phenanthrene carboxylic acid was verified by (CID) at collision energy of 25 eV. Product ions were recorded in a range of *m/z* 150 – 300, to check for loss of the carboxyl group and water molecule.

Enzyme Assays

Three liters of TRIP1 culture were separated from the HMN phase in a separation funnel in a glove box under N_2-atmosphere. After centrifugation for 30 min at 10,000 × *g* and 4°C, cell pellets were resuspended with 0.5 mL, 100 mM MOPS buffer (3-(N-morpholino)propanesulfonic acid, pH 7.3. The cells were collected in an Eppendorf cup and centrifuged again for 15 minutes at 13,000 × *g*, 4°C, and the pellet resuspended in MOPS buffer. The cells were opened in a French press (Thermo Electron, Waltham, USA) and centrifuged for 15 min at 4°C, 19,000 × g. Enzyme assays were composed according to Table 3. The assay was started by adding the substrate and incubated at 30°C and 900 rpm in a Thermomix Block (ThermoMixer® C, Eppendorf, Germany). Samples (40 µL) were mixed with double volume of methanol to stop the reaction, centrifuged again at 4°C, 13,000 × *g* for 15 minutes and transferred to LC-Vials.

	Carboxylase Assay	Ligase Assay	Reductase Assay
Phenanthrene	1 mM	1 mM	
2-PCA or 4-PCA			0.5 mM
$NaHCO_3$	5 mM		
ATP	5 mM	5 mM	5 mM
CoA-SH		1 mM	1 mM
DTT		2 mM	
$Na_2S_2O_4$			2 mM

Table 3: Composition of the enzyme assays. 2-PCA: 2-phenanthroic acid, 4-PCA: 4-phenanthroic acid, $NaHCO_3$: Sodium bicarbonate, ATP: Adenosine triphosphate, CoA-SH: Coenzyme-A, DTT: Dithiothreitol, $Na_2S_2O_4$: Sodium dithionite.

DNA extraction

DNA from the liquid culture was extracted as described in Lueders *et al.*, (2003) and Gabor *et al.*, (2003).

Metagenome sequencing

Illumina paired-end library preparation and sequencing

DNA (30 to 250 ng) was sheared by sonication to a 100- to 800-bp size range using a E210 Covaris instrument (Covaris, Inc., USA). Fragments were end-repaired, then 3`-adenylated, and Illumina adapters were added by using a NEB Next Sample Reagent Set (New England Biolabs). Ligation products were purified by Ampure XP (Beckmann Coulter) and DNA fragments (>200 bp) were PCR-amplified using Illumina adapter-specific primers and Platinum Pfx DNA polymerase (Invitrogen). The amplified library fragments were size-selected on 3% agarose gel around 300 bp. After library profile analysis with an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and qPCR quantification (MxPro, Agilent Technologies, USA) the library was sequenced using 101 base-length read chemistry in a paired-end flow cell V3 on the Illumina Hiseq2000 sequencer*//*(Illumina, USA, version RTA 1.13.48) in order to obtain overlapping reads and generate long reads of 180 bp.

Nextera Mate Paired library preparation and sequencing

The three mate pair libraries were prepared following the Nextera protocol (Nextera Mate Pair sample preparation kit, Illumina). Briefly, genomic DNA was simultaneously enzymatically fragmented and tagged with a biotinylated adaptor. Fragments were size selected (3-5, 5-8 Kb and 8-11 Kb) through regular gel electrophoresis and circularized overnight with a ligase. Linear, non-circularized fragments were digested and circularized DNA was fragmented to 300-1000 bp size range using Covaris E210. Biotinylated DNA was immobilized on streptavidin beads, end-repaired, then 3`-adenylated, and Illumina adapters were added. DNA fragments were PCR-amplified using

Illumina adapter-specific primers and then purified. Finally, libraries were quantified by qPCR and library profiles were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Each library was sequenced using 150 or 250 base-length read chemistry on a paired-end flow cell on the Illumina MiSeq (Illumina, USA).

The 8-11Kb library was sequenced using 151 base-length read chemistry in a paired-end flow cell on the Illumina MISEQ sequencer*//*(Illumina, USA, version RTA 1.18.54).

Processing of metagenomics shotgun reads

The raw reads were processed as in Adam *et al.* (2017) to reconstruct nearly complete genomes using the pre-assembly approach described in Gkanogiannis *et al.* (2016).

Acknowledgements:

T.B thanks Karine Labadie and Eric Mahieu for assistance with Illumina sequencing, and Genoscope's LABGeM team for developing and maintaining the MicroScope platform.

R.M. acknowledges funding from the ERC advanced grant EcOILogy no. 666952.

References:

Adam, I.K.U., Duarte, M., Pathmanathan, J., Miltner, A., Brüls, T., and Kästner, M. (2017) Microbial communities in pyrene amended soil–compost mixture and fertilized soil. *AMB Express* **7**: 7.

Annweiler, E., Materna, A., Safinowski, M., Kappler, A., Richnow, H.H., Michaelis, W., and Meckenstock, R.U. (2000) Anaerobic degradation of 2-methylnaphthalene by a sulfate-reducing enrichment culture. *Appl. Environ. Microbiol.* **66**: 5329–5333.

Annweiler, E., Michaelis, W., and Meckenstock, R.U. (2002) Identical ring cleavage products during anaerobic degradation of naphthalene, 2-methylnaphthalene, and tetralin indicate a new metabolic pathway. *Appl. Environ. Microbiol.* **68**: 852–858.

Annweiler, E., Richnow, H.H., Antranikian, G., Hebenbrock, S., Garms, C., Franke, S., et al. (2000) Naphthalene degradation and incorporation of naphthalene-derived carbon into biomass by the thermophile *Bacillus thermoleovorans*. *Appl. Environ. Microbiol.* **66**: 518–523.

Boll, M., Löffler, C., Morris, B.E.L., and Kung, J.W. (2014) Anaerobic degradation of homocyclic aromatic compounds via arylcarboxyl-coenzyme A esters: organisms, strategies and key enzymes. *Environ. Microbiol.* **16**: 612–627.

Cerniglia, C.E. (1993) Biodegradation of polycyclic aromatic hydrocarbons. *Curr. Opin. Biotechnol.* **4**: 331–338.

Cline, J.D. (1969) Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnol. Oceanogr.* **14**: 454–458.

Davidova, I.A., Gieg, L.M., Duncan, K.E., and Suflita, J.M. (2007) Anaerobic phenanthrene mineralization by a carboxylating sulfate-reducing bacterial enrichment. *ISME J.* **1**: 436–442.

Dong, X., Dröge, J., von Toerne, C., Marozava, S., McHardy, A.C., and Meckenstock, R.U. (2017) Reconstructing metabolic pathways of a member of the genus *Pelotomaculum* suggesting its potential to oxidize benzene to carbon dioxide with direct reduction of sulfate. *FEMS Microbiol. Ecol.* **93**: fiw254.

Dong, X., Greening, C., Brüls, T., Conrad, R., Guo, K., Blaskowski, S., et al. (2018) Fermentative Spirochaetes mediate necromass recycling in anoxic hydrocarbon-contaminated habitats. *ISME J.* (in press) https://doi.org/10.1038/s41396-018-0148-3.

Eberlein, C., Estelmann, S., Seifert, J., Von Bergen, M., Müller, M., Meckenstock, R.U., and Boll, M. (2013) Identification and characterization of 2-naphthoyl-coenzyme A reductase, the prototype of a novel class of dearomatizing reductases. *Mol. Microbiol.* **88**: 1032–1039.

Eberlein, C., Johannes, J., Mouttaki, H., Sadeghi, M., Golding, B.T., Boll, M., and Meckenstock, R.U. (2013) ATP-dependent/-independent enzymatic ring reductions involved in the anaerobic catabolism of naphthalene. *Environ. Microbiol.* **15**: 1832–1841.

Estelmann, S., Blank, I., Feldmann, A., and Boll, M. (2015) Two distinct old yellow enzymes are involved in naphthyl ring reduction during anaerobic naphthalene degradation. *Mol. Microbiol.* **95**: 162–172.

Fischer, E. (1883) Bildung von Methylenblau als Reaktion auf Schwefelwasserstoff. *Berichte der Dtsch. Chem. Gesellschaft* **16**: 2234–2236.

Gabor, E.M., Vries, E.J., Janssen, D.B., Keijzer-Wolters, A.C., van Elsa, sJ. D., Giovannoni, S.J., et al. (2003) Efficient recovery of environmental DNA for expression cloning by indirect extraction methods. *FEMS Microbiol. Ecol.* **44**: 153–163.

Galushko, A., Minz, D., Schink, B., and Widdel, F. (1999) Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium. *Environ. Microbiol.* **1**: 415–420.

Gkanogiannis, A., Gazut, S., Salanoubat, M., Kanj, S., and Brüls, T. (2016) A scalable assembly-free variable selection algorithm for biomarker discovery from metagenomes. *BMC Bioinformatics* **17**: 311.

Habe, H. and Omori, T. (2003) Genetics of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. *Biosci. Biotechnol. Biochem.* **67**: 225–243.

Horvath, R.S. (1972) Microbial co-metabolism and the degradation of organic compounds in nature.

Bacteriol. Rev. **36**: 146–155.

Johnsen, A.R., Wick, L.Y., and Harms, H. (2005) Principles of microbial PAH-degradation in soil. *Environ. Pollut.* **133**: 71–84.

Juhasz, A.L. and Naidu, R. (2000) Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene. *Int. Biodeterior. Biodegradation* **45**: 57–88.

Kiyohara, H. and Nagao, K. (1978) The catabolism of phenanthrene and naphthalene by bacteria. *J. Gen. Microbiol.* **105**: 69–75.

Koelschbach, J.S., Mouttaki, H., Pickl, C., Heipieper, H.J., Rachel, R., Lawson, P.A., and Meckenstock, R.U. (2017) *Rectinema cohabitans* gen. nov., sp. nov., a rod-shaped spirochaete isolated from an anaerobic naphthalene-degrading enrichment culture. *Int. J. Syst. Evol. Microbiol.* **67**: 1288–1295.

Kunapuli, U., Lueders, T., and Meckenstock, R.U. (2007) The use of stable isotope probing to identify key iron-reducing microorganisms involved in anaerobic benzene degradation. *ISME J.* **1**: 643–53.

Li, H. and Durbin, R. (2009) Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics* **25**: 1754–1760.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, A., et al. (2004) ARB: A software environment for sequence data. *Nucleic Acids Res.* **32**: 1363–1371.

Lueders, T., Manefield, M., and Friedrich, M.W. (2003) Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ. Microbiol.* **6**: 73–78.

McFarland, M.J. and Sims, R.C. (1991) Thermodynamic framework for evaluating PAH degradation in the subsurface. *Ground Water* **29**: 885–896.

Meckenstock, R.U., Annweiler, E., Michaelis, W., Richnow, H.H., and Schink, B. (2000) Anaerobic naphthalene degredation by a sulfate-reducing enrichment culture. *Appl. Environ. Microbiol.*

66: 2743–2747.

Meckenstock, R.U., Boll, M., Mouttaki, H., Koelschbach, J.S., Cunha Tarouco, P., Weyrauch, P., et al. (2016) Anaerobic degradation of benzene and polycyclic aromatic hydrocarbons. *J. Mol. Microbiol. Biotechnol.* **26**: 92–118.

Meckenstock, R.U. and Mouttaki, H. (2011) Anaerobic degradation of non-substituted aromatic hydrocarbons. *Curr. Opin. Biotechnol.* **22**: 406–414.

Meckenstock, R.U., Safinowski, M., and Griebler, C. (2004) Anaerobic degradation of polycyclic aromatic hydrocarbons. *FEMS Microbiol. Ecol.* **49**: 27–36.

Menzie, C.A., Potocki, B.B., and Santodonato, J. (1992) Ambient concentrations and exposure to carcinogenic PAHs in the environment. *Environ. Sci. Technol.* **26**: 1278–1284.

Mouttaki, H., Johannes, J., and Meckenstock, R.U. (2012) Identification of naphthalene carboxylase as a prototype for the anaerobic activation of non-substituted aromatic hydrocarbons. *Environ. Microbiol.* **14**: 2770–2774.

Parks, D., Imelfort, M., and Skennerton, C. (2015) CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome* **25**: 1043–1055.

Pernthaler, J., Glöckner, F.O., Schönhuber, W., and Amann, R. (2001) Fluorescence in situ hybridization with rRNA-targeted oligonucleotide probes. *Methods Microbiol.* **30**: 207–226.

Safinowski, M. and Meckenstock, R.U. (2004) Enzymatic reactions in anaerobic 2-methylnaphthalene degradation by the sulphate-reducing enrichment culture N47. *FEMS Microbiol. Lett.* **240**: 99–104.

Samanta, S.K., Singh, O. V., and Jain, R.K. (2002) Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. *Trends Biotechnol.* **20**: 243–248.

Schnell, S., Bak, F., and Pfennig, N. (1989) Anaerobic degradation of aniline and dihydroxybenzenes by newly isolated sulfate-reducing bacteria and description of *Desulfobacterium anilini*. *Arch. Microbiol.* **152**: 556–563.

Suzuki, D., Li, Z., Cui, X., Zhang, C., and Katayama, A. (2014) Reclassification of

Desulfobacterium anilini as *Desulfatiglans anilini* comb. nov. within *Desulfatiglans* gen. nov., and description of a 4-chlorophenol-degrading sulfate-reducing bacterium, *Desulfatiglans parachlorophenolica* sp. *Int. J. Syst. Evol. Microbiol.* **64**: 3081–3086.

Tabatabai, M.A. (1974) A rapid method for determination of sulfate in water samples. *Environ. Lett.* **7**: 237–243.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**: 2731–2739.

Vallenet, D., Belda, E., Calteau, A., Cruveiller, S., Engelen, S., Lajus, A., et al. (2013) MicroScope - an integrated microbial resource for the curation and comparative analysis of genomic and metabolic data. *Nucleic Acids Res.* **41**: D636–D647.

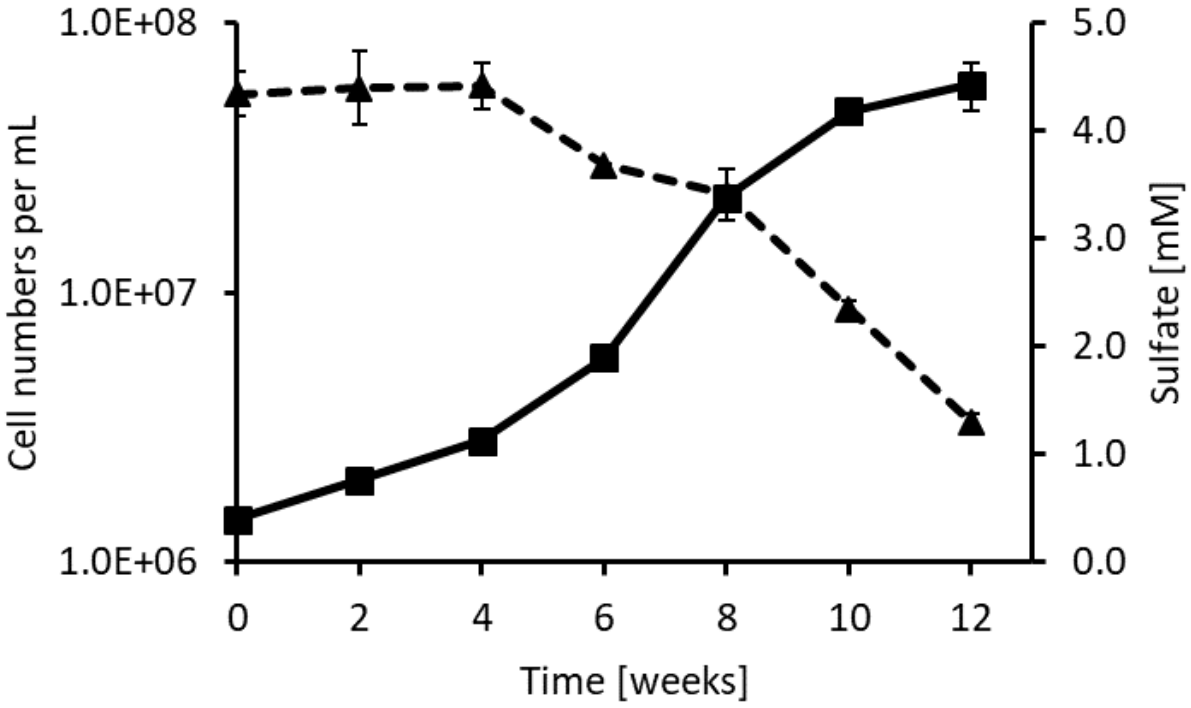
Vallenet, D., Labarre, L., Rouy, Z., Barbe, V., Bocs, S., Cruveiller, S., et al. (2006) MaGe: a microbial genome annotation system supported by synteny results. *Nucleic Acids Res.* **34**: 53–65.

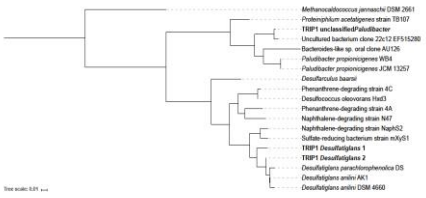
Weyrauch, P., Zaytsev, A. V., Stephan, S., Kocks, L., Schmitz, O.J., Golding, B.T., and Meckenstock, R.U. (2017) Conversion of cis-2-carboxycyclohexylacetyl-CoA in the downstream pathway of anaerobic naphthalene degradation. *Environ. Microbiol.* **19**: 2819–2830.

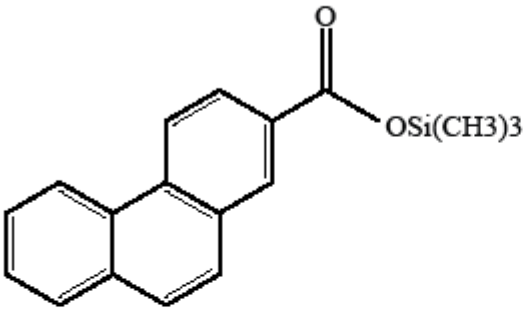
Widdel, F. and Bak, F. (1992) Gram-negative mesophilic sulfate-reducing bacteria. In, Balows A., Trüper H.G., Dworkin M., Harder W.,S.K. (ed), *The prokaryotes*. Springer, New York, NY, pp. 3352–3378.

Widdel, F., Kohring, G., and Mayer, F. (1983) Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. *Arch. Microbiol.* **129**: 395–400.

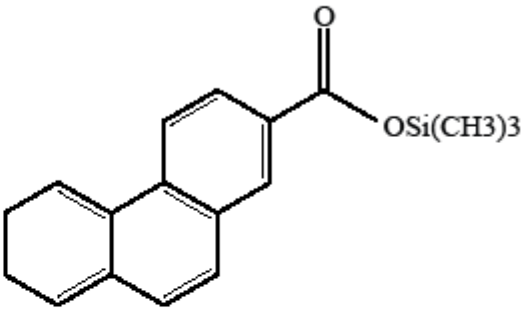
Zhang, X. and Young, L.Y. (1997) Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. *Appl. Environ. Microbiol.* **63**: 4759–4764.



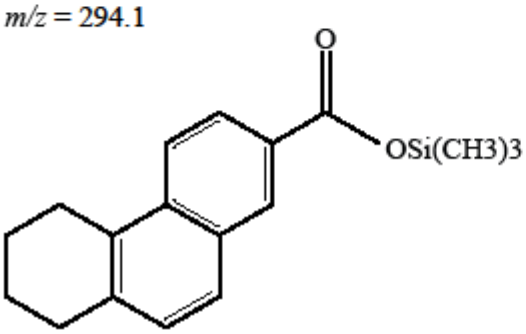




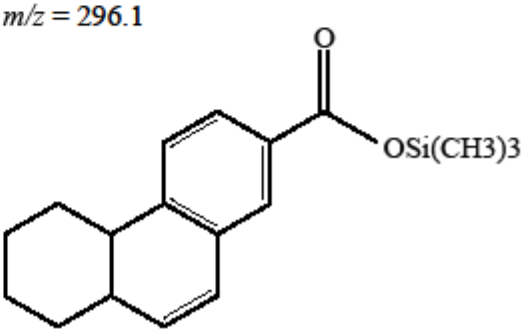
Sylilated 2-phenanthroic acid
Chemical Formula: $C_{18}H_{18}O_2Si^+$
m/z = 294.1



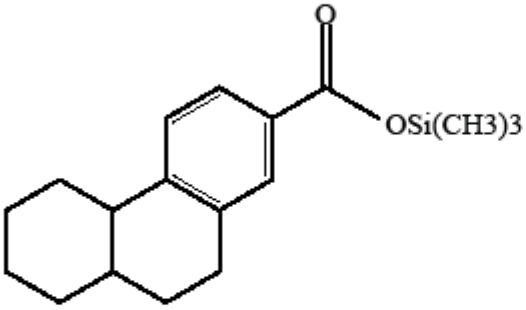
Sylilated dihydro-2-phenanthroic acid
Chemical Formula: $C_{18}H_{20}O_2Si^+$
m/z = 296.1



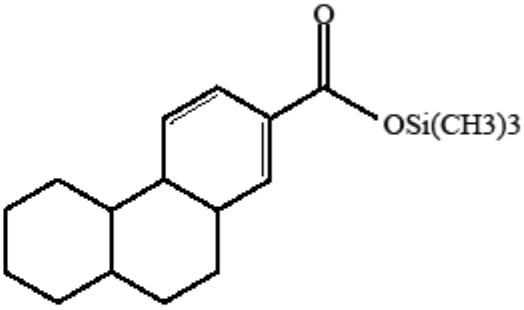
Sylilated tetrahydro-2-phenanthroic acid
Chemical Formula: $C_{18}H_{22}O_2Si^+$
m/z = 298.1



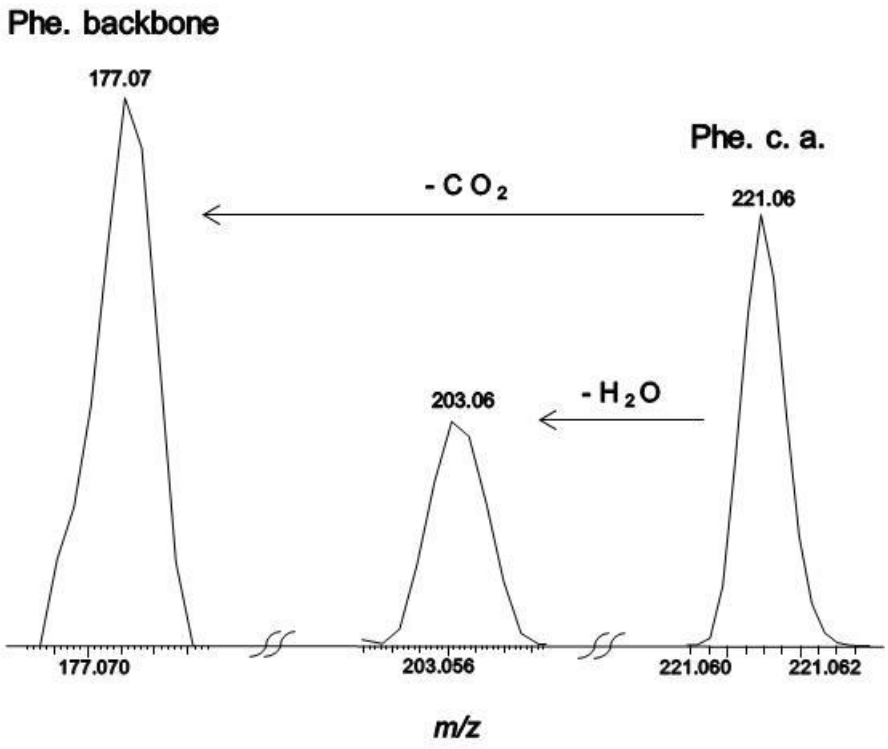
Sylilated hexahydro-2-phenanthroic acid
Chemical Formula: $C_{18}H_{24}O_2Si^+$
m/z = 300.1

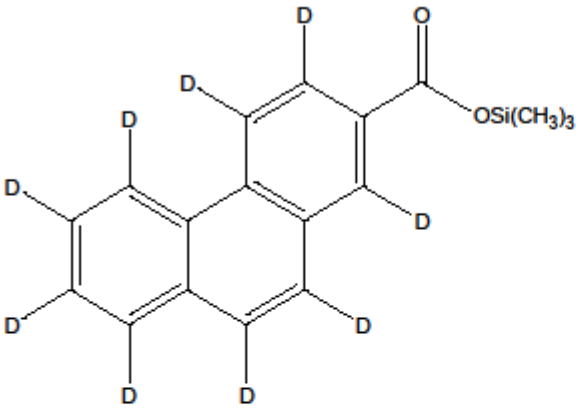


Sylilated octahydro-2-phenanthroic acid
Chemical Formula: $C_{18}H_{26}O_2Si^+$
m/z = 302.1

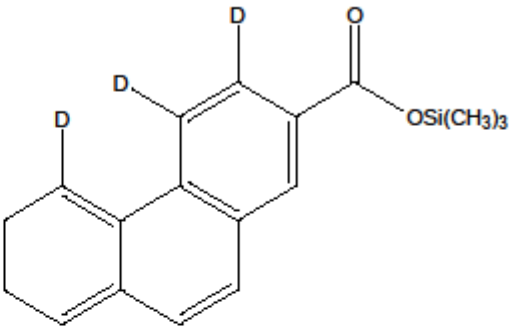


Sylilated decahydro-2-phenanthroic acid
Chemical Formula: $C_{18}H_{28}O_2Si^+$
m/z = 304.1

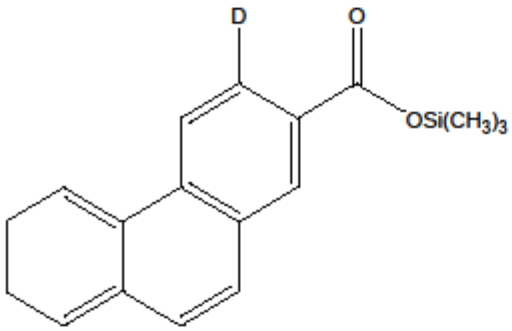




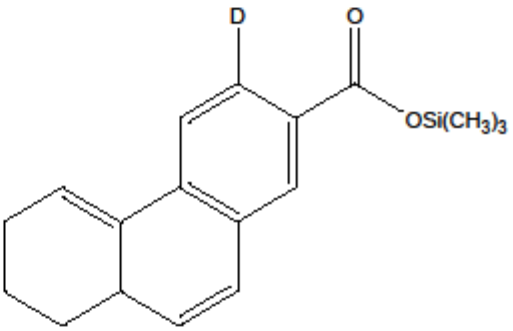
Silylated 2-phenanthroic acid (D_{10})
m/z= 303.16



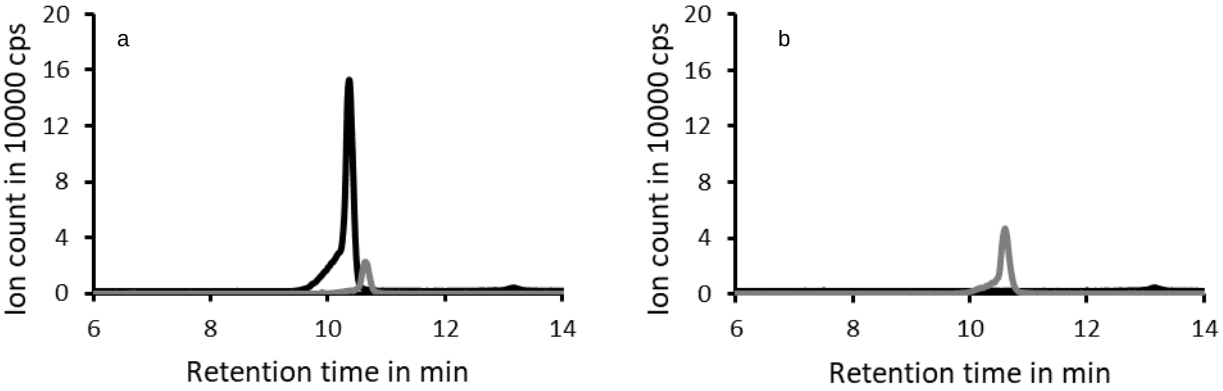
Silylated 6,7-dihydro-2-phenanthroic acid (D_3)
m/z= 299.14

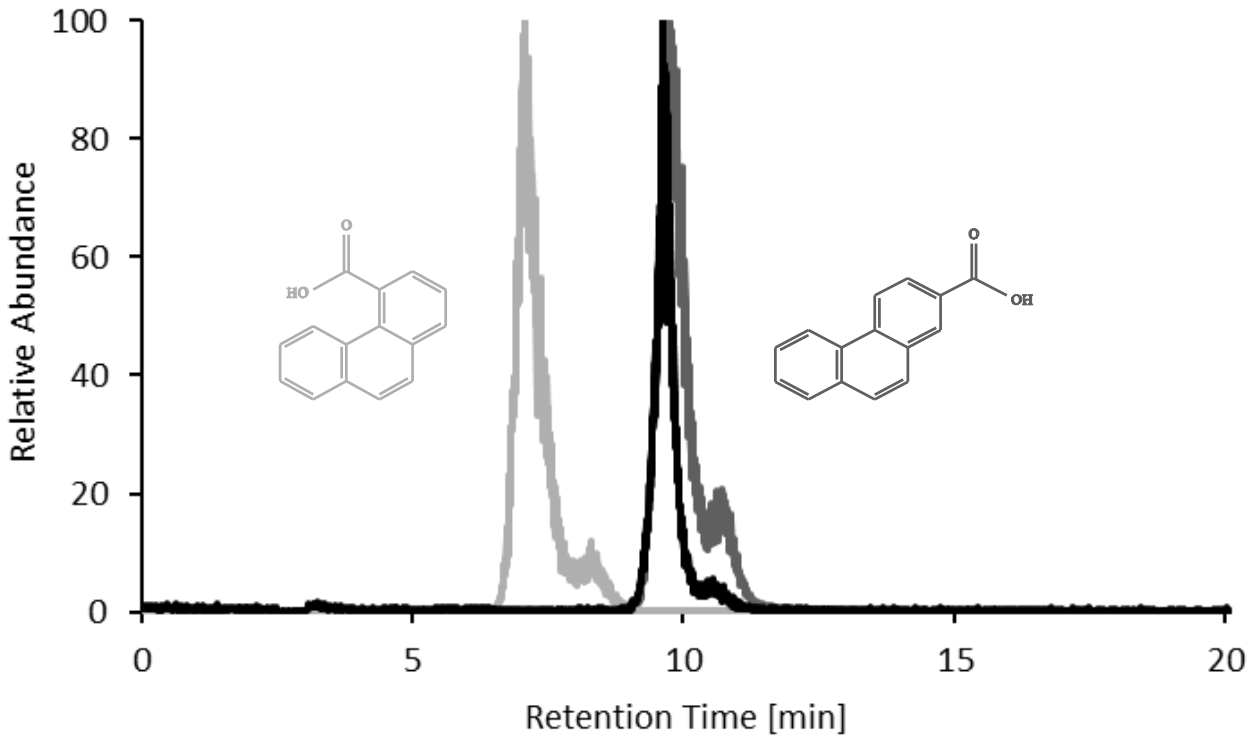


Silylated 6,7-dihydro-2-phenanthroic acid (D_1)
m/z= 297.13



Silylated 6,7,8,9-tetrahydro-2-phenanthroic acid (D_1)
m/z= 299.14





Substrate	Total cell density[a] [cells/ml]	T-RFLP fragment length bp [b]	Assignment to organism
Phenanthrene (positive control)	6.8 x 10^6 (+)	**53**; 74	*Desulfatiglans*-1
Naphthalene	*(*)*	–	
Acenaphthene	*(*)*	–	
Anthracene	*(*)*	–	
Fluoranthene	*(*)*	–	
Benzene	7.0 x 10^6 (+)	53; 74; **515**	*Desulfatiglans*-2
Benzoic acid	5.1 x 10^7 (+)	53; 74; **515**	*Desulfatiglans*-2
1-Naphthoic acid	2.3 x 10^6 (+)	*(*)*	
2-Naphthoic acid	2.7 x 10^6 (+)	**53**; 74; 97	*Desulfatiglans*-1, unclassified bacterium
1,2,3,4-Tetrahydro-2-naphthoic acid	5.3 x 10^4 (-)		
5,6,7,8-Tetrahydro-2-naphthoic acid	5.2 x 10^4 (-)		
2-Phenanthroic acid	1.3 x 10^7 (+)	53; **404**	unknown
4-Phenanthroic acid	7.1 x 10^5 (+)	**53**; 74	*Desulfatiglans*-1
Terephthalic acid	8.6 x 10^6 (+)	**156**	unknown

Table 1: Substrate utilization by culture TRIP1. a) Growth was evaluated as positive (+) if the cell count in the second transfer increased more than ten-fold compared to the control without terminal electron acceptor (9.8 x 10^4), as measured by flow cytometry, (-) bacterial growth lower than ten-fold compared to negative control or no detectable growth, (*) PCR amplification not possible. b) Fragment length in bold describes the dominant peaks in the TRFLP analysis.

Organism	Marker lineage	# genomes	# markers	# uniquely found markers	Completeness	Contamination
Dominant *Desulfobacteraceae* organism	Deltaproteobacteria (UID3216)	83	247	244	99.35	0.97
Geobacter-like organism	Bacteria (UID2495)	2993	147	143	99.94	5.49
Paludibacter-like organism	Bacteria (UID2569)	434	278	266	95.61	0.27
Spirochaete	Bacteria (UID2495)	2993	141	140	98.85	0.00

Table 2

Genome completion estimates of metagenomic "bins" constructed from culture TRIP1. Strain heterogeneity values (not shown in the Table) were 0.00 for all the genomes.

	Carboxylase Assay	Ligase Assay	Reductase Assay
Phenanthrene	1 mM	1 mM	
2-PCA or 4-PCA			0.5 mM
$NaHCO_3$	5 mM		
ATP	5 mM	5 mM	5 mM
CoA-SH		1 mM	1 mM
DTT		2 mM	
$Na_2S_2O_4$			2 mM

Table 3

Composition of the enzyme assays. 2-PCA: 2-phenanthroic acid, 4-PCA: 4-phenanthroic acid, $NaHCO_3$: Sodium bicarbonate, ATP: Adenosine triphosphate, CoA-SH: Coenzyme-A, DTT: Dithiothreitol, $Na_2S_2O_4$: Sodium dithionite.