Front. Environ. Sci. Eng. 2019, 13(5): 80
https://doi.org/10.1007/s11783-019-1164-x

RESEARCH ARTICLE

Anaerobic phenanthrene biodegradation with four kinds of electron acceptors enriched from the same mixed inoculum and exploration of metabolic pathways

Zuotao Zhang[1], Chongyang Wang[1,2], Jianzhong He[3], Hui Wang (✉)[1]

1 State Key Joint Laboratory of Environment Simulation and Pollution Control, School of Environment, Tsinghua University, Beijing 100084, China
2 Miami College, Henan University, Kaifeng 475000, China
3 Department of Civil and Environmental Engineering, National University of Singapore, Singapore 117576, Singapore

HIGHLIGHTS

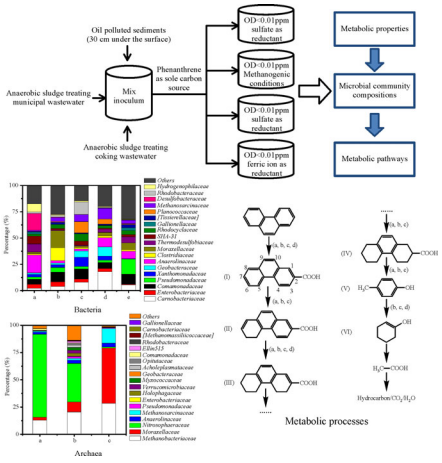
- Anaerobic phenanthrene biodegradation enriched process was described in detail.
- The enriched bacterial communities were characterized under four redox conditions.
- The enriched archaeal communities were stated under high percentage conditions.
- Relatively intact pathways of anaerobic phenanthrene biodegradation were proposed.

ARTICLE INFO

Article history:
Received 9 May 2019
Revised 5 September 2019
Accepted 5 September 2019
Available online 13 October 2019

Keywords:
Phenanthrene
Anaerobic biodegradation
Bacterial populations
Archaea populations
Metabolic pathway

GRAPHIC ABSTRACT



ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are widespread and persistent contaminants worldwide, especially in environments devoid of molecular oxygen. For lack of molecular oxygen, researchers enhanced anaerobic zones PAHs biodegradation by adding sulfate, bicarbonate, nitrate, and iron. However, microbial community reports of them were limited, and information of metabolites was poor except two-ring PAH, naphthalene. Here, we reported on four phenanthrene-degrading enrichment cultures with sulfate, bicarbonate, nitrate, and iron as electron acceptors from the same initial inoculum. The high-to-low order of the anaerobic phenanthrene biodegradation rate was the nitrate-reducing conditions>sulfate-reducing conditions>methanogenic conditions>iron-reducing conditions. The dominant bacteria populations were *Desulfobacteraceae*, *Anaerolinaceae,* and *Thermodesulfobiaceae* under sulfate-reducing conditions; *Moraxellaceae*, *Clostridiaceae,* and *Comamonadaceae* under methanogenic conditions; *Rhodobacteraceae*, *Planococcaceae,* and *Xanthomonadaceae* under nitrate-reducing conditions; and *Geobacteraceae*, *Carnobacteriaceae,* and *Anaerolinaceae* under iron-reducing conditions, respectively. Principal component analysis (PCA) indicated that bacteria populations of longtime enriched cultures with four electron acceptors all obtained significant changes from original inoculum, and bacterial communities were similar under nitrate-reducing and iron-reducing conditions. Archaea accounted for a high percentage under iron-reducing and methanogenic conditions, and *Methanosarcinaceae* and *Methanobacteriaceae,* as well as *Methanobacteriaceae,* were the dominant archaea populations under iron-reducing and methanogenic conditions. The key steps of phenanthrene biodegradation under four reducing conditions were carboxylation, further ring system reduction, and ring cleavage.

© Higher Education Press and Springer-Verlag GmbH Germany, part of Springer Nature 2019

✉ Corresponding author
E-mail: wanghui@tsinghua.edu.cn

1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are major components of coal, creosote and crude oil that form through the chemical conversion of biomass in sedimentary systems or secondary metabolites of organisms. A large number of PAHs have been released into the environment due to natural and anthropic activities, such as a volcanic explosion, crude oil spillage, and gasoline leakage. As frequent environmental contaminants, biodegradation of PAHs had been known for several decades (Smith, 1990; Li et al., 2017). In the early days, some researchers indicated that PAHs could not be biodegraded anaerobically (Hambrick et al., 1980; Bauer and Capone, 1985). Whereas naphthalene oxidized by adding sulfate, nitrate, bicarbonate, and Fe (III) under anaerobic conditions was observed in the following days (Mihelcic and Luthy, 1988; Coates et al., 1996;Langenhoff et al., 1996; Galushko et al., 1999; Berdugo-Clavijo et al., 2012). Sulfate, nitrate, bicarbonate, and Fe (III) were reported to enhance PAHs biodegradation in anaerobic zones efficiently (Trably et al., 2003; Xu et al., 2015; Müller et al., 2017). Anthracene, acenaphthene, fluorene, phenanthrene, and even pyrene were also reported to be biodegradable in the absence of oxygen (Chang et al., 2003). However, major results only focused on the ability of biodegradation, promoting effect, microbial changes with naphthalene as sole carbon sources, and anaerobic naphthalene biodegradation processes.

Anaerobic naphthalene biodegradation was initially reported to be completed by *δ-proteobacteria* under sulfate-reducing conditions (Galushko et al., 1999). Two marine strains, NaphS2 and NaphS3, were reported to degrade naphthalene under sulfate-reducing conditions (Galushko et al., 1999; Musat et al., 2009). No studies had provided credibly pure cultures under other reducing conditions. Bacterial communities involved in anaerobic naphthalene biodegradation were enriched, and their community structures characterized by many researchers with different electron acceptors (Kleemann and Meckenstock, 2011; Berdugo-Clavijo et al., 2012; Kümmel et al., 2015; Martirani-Von Abercron et al., 2016). Methylation or carboxylation had been identified as the initial step of anaerobic naphthalene biodegradation (Zhang and Young, 1997; Safinowski and Meckenstock, 2006). The downstream metabolic pathways of naphthalene, such as ring reduction and ring cleavage had been reported in pure culture or the coculture under sulfate-reducing conditions (Meckenstock et al., 2000; Weyrauch et al., 2017).

So far, microbial consortia capable of anaerobic biodegradation of higher molecular weight polycyclic aromatic hydrocarbons, such as phenanthrene, are limited. Phenanthrene ($C_{14}H_{10}$) is one of the priority pollutant PAHs and has long been a model PAH for the study of aerobic PAH biodegradation, however, in anaerobic phenanthrene biodegradation, only the community structures under sulfate-reducing and methanogenic conditions had been characterized for thrice and once (Zhang and Young, 1997; Davidova et al., 2007; Zhang et al., 2012; Himmelberg et al., 2018). Carboxylation was identified as the initial step of anaerobic phenanthrene biodegradation by sulfate-reducing cultures (Zhang and Young, 1997; Davidova et al., 2007; Himmelberg et al., 2018). Other enriched cultures for anaerobic phenanthrene biodegradation were not characterized, and their metabolic processes had not been illustrated.

Here, we reported on four anaerobic phenanthrene-degrading cultures enriched from the same origin under sulfate-reducing, methanogenic, nitrate-reducing, and iron-reducing conditions. Bacterial community structures under all reducing conditions and archaeal community structures under methanogenic and iron-reducing conditions were characterized. Besides, anaerobic phenanthrene biodegradation pathways were proposed based on metabolite analyses under these four reducing conditions.

2 Materials and methods

2.1 Reactors operation for the enrichment of anaerobic phenanthrene biodegradation cultures

Enrichment experiments were conducted in four 1.5 L reactors equipped with dissolved oxygen (DO) probes, pH probes, temperature probes, and 0.5 L gasbags. Petroleum-contaminated soil, coking sludge and domestic sludge with a volatile suspended solids (VSS) ratio (g: g: g) of 5: 1: 1 were provided as initial inoculum, which was the same as described previously (Ye et al., 2018). Phenanthrene, mineral salt media, and trace element solutions were added as described previously (Yuan and Chang, 2007; Obi et al., 2017). The initial phenanthrene concentrations were controlled between 20 and 30 mg/L, and 20 mM $NaHCO_3$, Na_2SO_4, $NaNO_3$, and ferric citrate were provided as electron acceptors. The culture dissolved oxygen (DO) in the reactors was maintained below 0.01 ppm by bubbling with N_2/H_2 (99:1, v/v) gas and automatically measured online. The cultures pH and temperature were controlled at 7–7.6 and 32°C±1°C, respectively. The stirring speed of the electric blender was set at 120 r/min to produce shear force and improve nutrition transfer. The four reactors were wrapped with aluminum foil to prevent photolysis.

2.2 Phenanthrene and ion concentration measurement

Samples were regularly taken from the bottom of the reactors by extruding gas from gasbags. The phenanthrene concentration was analyzed as described previously (Fang et al., 2016), and was then confirmed by Gas Chromatography (GC) as described previously (Feng and Zhu, 2018). SO_4^{2-}, S^{2-}, NO_3^-, NO_2^-, Fe^{3+}, Fe^{2+}, and Volatile

Suspended Solids (VSS) were measured using the standard methods set by the American Public Health Association (APHA, 1998). All experiments were conducted in triplicate.

2.3 Fluorescence In Situ Hybridization (FISH) for enrichment cultures

Samples to be analyzed were washed with a phosphate buffer solution in triplicate and fixed in 4% paraformaldehyde (PFA) for 2–6 h at 4°C. Then, 10 μL of consortia were drawn and placed on the glass slide; 50%, 80%, and 100% ethanol were used to dehydrate the consortia. Hybridization was performed at 46°C for 90 min in a wet environment by adding 1 μL of a 16S rRNA-targeted oligonucleotide probe and 9 μL of a hybridization buffer mixture. The probes EUB338-I GCTGCCTCCCGTAGGAGT, EUB338-II GCAGCCACCCGTAGGTGT, and EUB338-III GCTGCCACCCGTAGGTGT labeled with Cy5 at the 5′ end were applied for targeting bacteria, and the probe ARCH915 GTGCTCCCCCGCCAATTCCT labeled with Cy3 at the 5′ end was used to target archaea (Amann and Fuchs, 2008). The formamide concentrations in the hybridization buffer and washing buffer were used as described previously (Luo et al., 2014; Xu et al., 2015). Images were obtained with an LSM 50 META laser scanning microscope acquisition system (Zeiss, LSM510 META, Germany) controlled by confocal software v3.2 (Zeiss, Germany). The percentages of archaea accounting in the total population were calculated using the standard software package of the instrument (version 4.0).

2.4 Microbial community analysis

Enrichment cultures were subjected to DNA extraction, 16S rRNA gene amplification, and subsequent microbial community analysis by Illumina sequencing. In this study, DNA was extracted from the original inoculation mixture and 244-day enrichment cultures using a commercial kit (FastDNA® Spin Kit for Soil) according to the manufacturer's instructions. The primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) were used to amplify the V4 region of the bacterial 16S rDNA gene (Yarza et al., 2014). The primers Arch519F (CAGYMGCCRCGGKAAHACC) and Arch806R (GGACTACNSGGGTMTCTAAT) were used to amplify the archaea (Luo et al., 2015). The PCR amplification conditions were as follows: 95°C, 3 min; 25 cycles of 95°C 30 s, 55°C 45 s, 72°C 90 s; 72°C 10 min; final hold at 4°C until use. PCR amplification was repeated in triplicate for each sample, and the mixture was purified via gel recovery with an AxyPrepDNA PCR purification kit. The PCR products were identified by Illumina 2500, and a HISeq library was constructed. Sequences were quality controlled, and those with similarity over 97% were grouped as an operational taxonomic unit (OTU). The classification was completed by greengenes (version 13.8) and a manual BLAST. The 16S rRNA gene sequences from this study were submitted to GenBank under SRA accession number SRS2645772-SRS2645776 (bacterial sequences), and SRS2645881-SRS2645883 (archaeal sequences). Meantime, bacterial families of enriched cultures with four electron acceptors were analyzed by PCA.

2.5 Metabolic products analysis by GC-MS

Five-hundred milliliter enrichment cultures of each reducing conditions were centrifuged, and the supernatants transferred to a 2 L separating funnel. An equal volume of methylene chloride was added to the supernatant, the pH of the supernatant was adjusted to 12 with 1 M NaOH for 15 min, and then adjusted to 2 with 1 M HCl, the metabolic products were extracted by manual shaking for 5 min. The extract was transferred to a 5 L distilling flask, and another more two times extract were performed as above. The extract was transferred to the same distilling flask and then concentrated by rotary evaporation and nitrogen stripping until a dry pellet was formed. 0.1 mL acetonitrile and 0.1 mL methylene chloride were added to dissolve the dried pellet; the resulting solution was transferred to 2 mL vials. The dissolved pellet was derivatized with 0.1 mL BSTFA-0.1% TMS at 60°C for 30 min. Nitrogen stripping was performed again to dry the derivative sample, and the dried sample was finally dissolved in 1 mL methylene chloride.

Additionally, 2-phenanthroic acid standard diluted into 500 mL of pure water was extracted and derived as above.

Two μL derivative sample was injected onto a GC-mass spectrometer (MS) coupled to a mass-selective detector (Agilent 7890A-5975C, Wilmington, USA). The column for GC-MS was 30 m by 0.25 mm (inner diameter) coupled with phase DB-5 MS with a film thickness of 0.25 mm. The injector temperature was 280°C, and the column temperature started at 80°C for 5 min, increased to 300°C at 4°C/min for the metabolic products from phenanthrene anaerobic biodegradation and finally held at 300°C for 10 min.

3 Results

3.1 Microbial activity and growth of enrichments

For the mixed culture enriched for 244 days, the phenanthrene concentrations at each period were measured under sulfate-reducing, methanogenic, nitrate-reducing, and iron-reducing conditions. Anaerobic phenanthrene biodegradation rate is initially high, then rapid decreasing, and finally increased and stabilized under all of the reducing conditions. The anaerobic phenanthrene biodegradation speed stabilized between 7 and 8 μM/d under the

sulfate-reducing conditions, 4.5–5.2 μM/d under the methanogenic conditions, 8.1–8.5 μM/d under the nitrate-reducing conditions, and 2.7–2.75 μM/d under the iron-reducing conditions from period 12 to period 14. The high-to-low order of anaerobic phenanthrene biodegradation rates for all of the reducing conditions was nitrate-reducing conditions>sulfate-reducing conditions>methanogenic conditions>iron-reducing conditions (Fig. 1).

Sulfate, nitrate and ferriferous ions were depleted with anaerobic phenanthrene biodegradation, and significant amounts of sulfide ions, ferrous ions, and few nitrite produced under different reducing conditions (Fig. 2).

The mole ratio of sulfate ion and phenanthrene depletion changed from 36.7 to 8.3 and stabilized between 8 and 8.7 from period 8 to 14 under the sulfate-reducing conditions, nitrate decrease coupled with the anaerobic phenanthrene degradation mole ratio changed from 113 to 23 and stabilized between 23 and 37 from period 10 to 14 under the nitrate-reducing conditions, and ferric ion and phenanthrene depletion mole ratio changed from 96 to 67 and stabilized between 68 and 75 from period 10 to 14 under the iron-reducing conditions (Table 1).

VSS under all of the reducing conditions showed a trend of rapid increase, fast decrease, and slowly increase. The rapid increase might be caused by the remaining organic carbon in the initial inoculum. The fast decrease might result from the selective function of phenanthrene and anaerobic environment. The slow growth period represented the true growth of microbes for anaerobic phenanthrene biodegradation. VSS increased by 16.1% under the sulfate-reducing conditions, 21.4% under the methanogenic conditions, 25.4% under the nitrate-reducing conditions, and 34.1% under the iron-reducing conditions from day 153 to day 244 (Table 2). The biological proliferation of anaerobic phenanthrene biodegradation was slow over the entire period. The anaerobic phenanthrene biodegradation activities based on biomass were 3.9–4.1 μM/(d·g VSS) under the sulfate-reducing conditions, 2.5–2.7 μM/(d·g VSS) under the methanogenic conditions, 3.6–3.8 μM/(d·g VSS) under the nitrate-reducing conditions, and 0.94–1.1 μM/(d·g VSS) under the iron-reducing conditions from period 12 to period 14. The high-to-low order of anaerobic phenanthrene biodegradation activities based on biomass for all of the reducing conditions was sulfate-reducing conditions> nitrate-reducing conditions>methanogenic conditions >iron-reducing conditions.

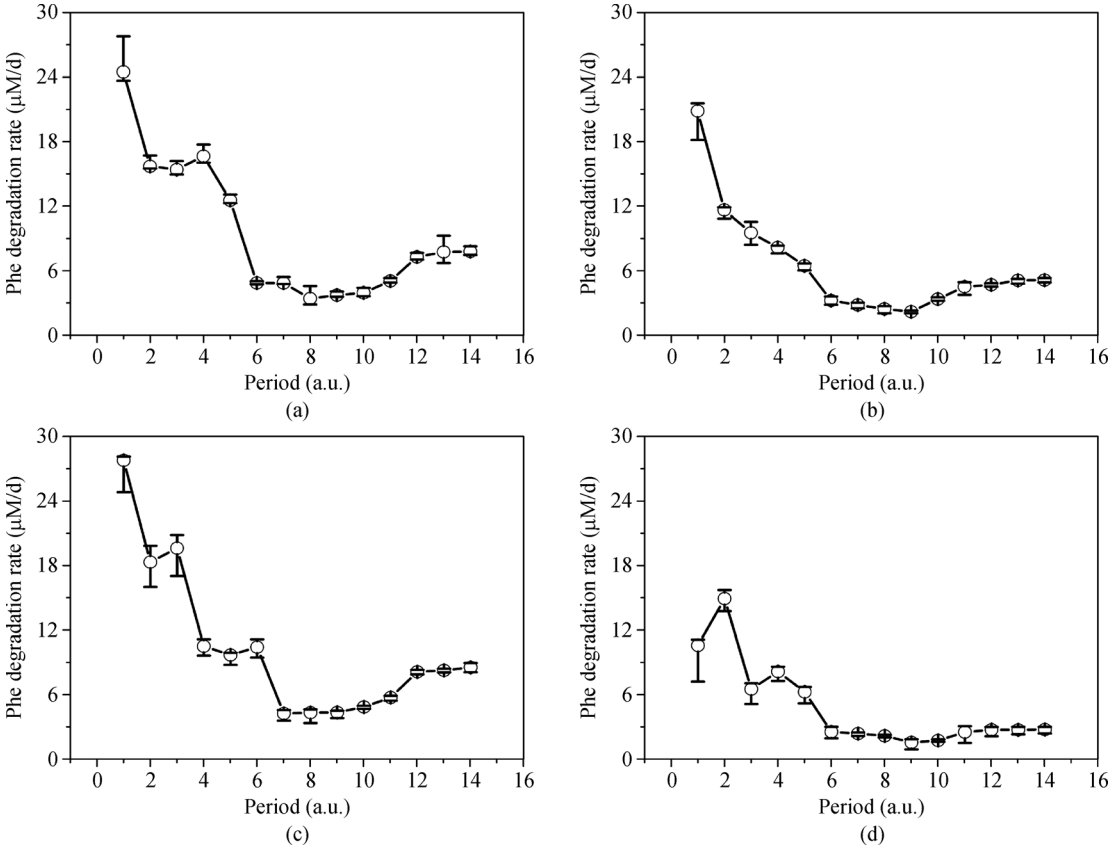


Fig. 1 Anaerobic phenanthrene biodegradation under various redox conditions. (a) sulfate-reducing conditions; (b) methanogenic conditions; (c) nitrate-reducing conditions; (d) iron-reducing conditions. Period 1: day 0–3; period 2: day 3–9; period 3: day 12–17; period 4: day 19–32; period 5: day 35–45; period 6: day 50–60; period 7: day 66–75; period 8: day 76–85; period 9: day 86–106; period 10: day 125–153; period 11: day 154–174; period 12: day 176–198; period 13: day 200–222 and period 14: day 223–244.

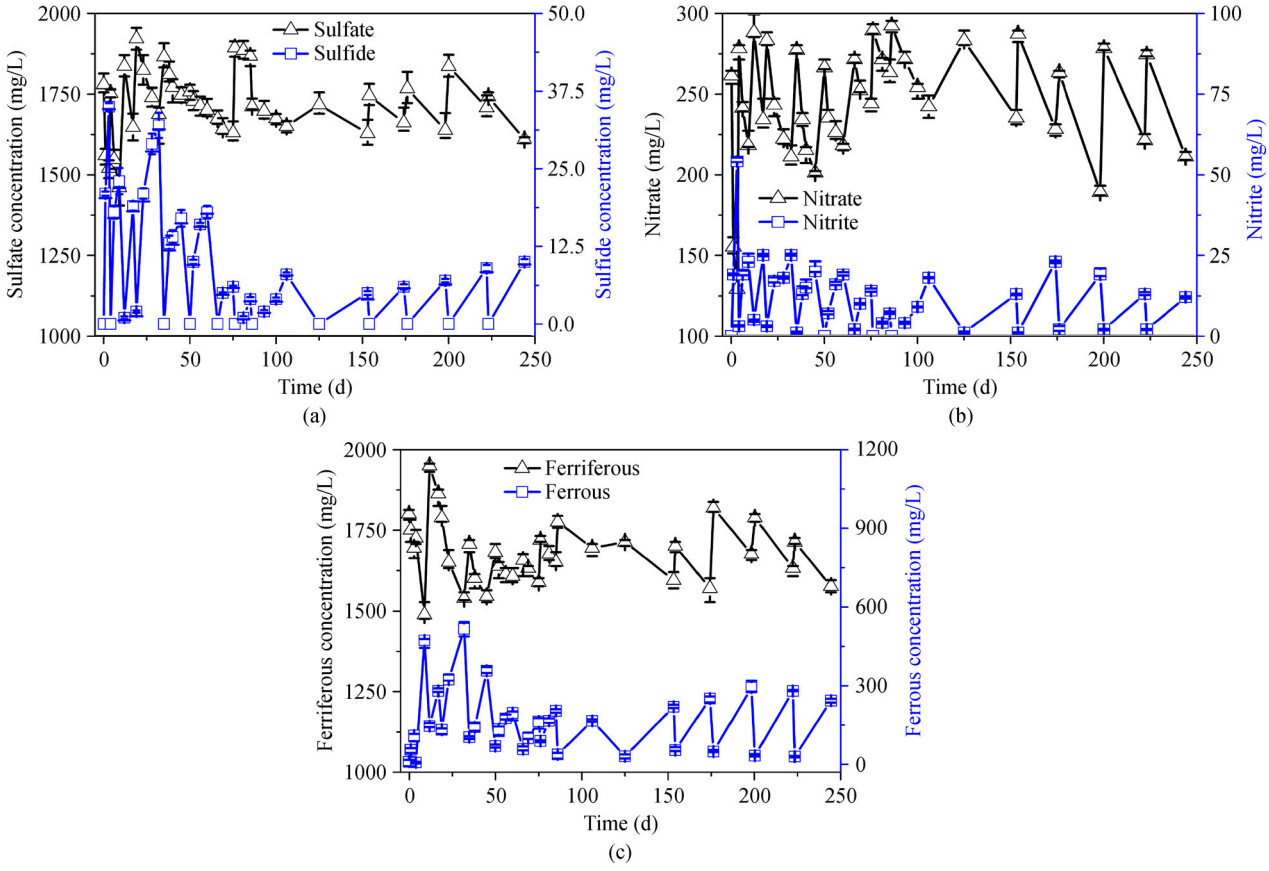


Fig. 2 Ion conversion under partial redox conditions. (a) sulfate-reducing conditions; (b) nitrate-reducing conditions; (c) iron-reducing conditions.

Table 1 Overview of the ion and phenanthrene consumption mole ratio

$\Delta SO_4^{2-}/\Delta Phe$	$\Delta NO_3^{-}/\Delta Phe$	$\Delta Fe^{3+}/\Delta Phe$	Period
36.69	113.30	95.65	1
32.25	39.04	75.80	2
25.55	39.36	76.80	3
11.28	37.79	67.36	4
9.80	56.21	74.24	5
12.17	32.98	84.58	6
9.20	45.84	82.70	7
7.90	43.26	90.74	8
8.50	41.53	75.32	9
8.36	25.31	69.94	10
8.66	37.02	74.40	11
8.31	29.63	68.50	12
8.26	22.48	74.91	13
8.27	25.15	68.00	14

3.2 Microbial community structures analysis

Bacteria and archaea had been reported to biodegrade PAHs in anaerobic environments (Tor and Lovley, 2001; Musat et al., 2009). FISH was applied to detect the archaea and bacteria that participate in anaerobic phenanthrene biodegradation under all reducing conditions (Fig. 3). Red fluorescence excitation represented bacteria, and the green fluorescence excitation represented archaea, and the proportions of archaea under different conditions were different. The archaea percentage was 26%±8% in the original inoculum, 3%±2% in the sulfate-reducing conditions, 17%±6% in the methanogenic conditions, 8%±4% in the nitrate-reducing conditions, and 24%±9% in the iron-reducing conditions after 244 days enrichment.

In this study, microbial community structures were analyzed by high-throughput sequencing to identify the dominant microbes under various redox conditions. Analysis of the microbial community structure via sequencing of the V4 regions of the bacterial 16S rDNA gene revealed that 82.5% under the sulfate-reducing conditions, 72.2% under the methanogenic conditions, 85.3% under the nitrate-reducing conditions, and 80% under the iron-reducing conditions, as well as 68.9% of phylotypes in the original inoculum, could be identified to the family level. The families identified are summarized in Fig. 4, and the bacterial families that were highly abundant

Table 2 Overview of VSS (g/L) changes in this study

Sample day	Sulfate-reducing conditions	Methanogenic conditions	Nitrate-reducing conditions	Iron-reducing conditions
Day 0	1.43	1.45	1.46	1.42
Day 9	1.86	1.76	1.99	1.79
Day 32	1.94	1.79	1.83	1.83
Day 45	1.84	1.82	1.75	1.86
Day 60	1.73	1.67	1.74	1.92
Day 106	1.65	1.58	1.78	2.05
Day 153	1.68	1.54	1.89	2.17
Day 174	1.73	1.64	2.04	2.31
Day 198	1.81	1.77	2.11	2.49
Day 222	1.88	1.85	2.23	2.68
Day 244	1.95	1.96	2.37	2.91

under the sulfate-reducing conditions were *Anaerolineaceae* (16.9% relative abundance), *Desulfobacteraceae* (16% relative abundance) and *Thermodesulfobiaceae* (7.7% relative abundance); *Moraxellaceae* (16.1% relative abundance), *Clostridiaceae* (12.4% relative abundance) and *Comamonadaceae* (9.3% relative abundance) under the methanogenic conditions; *Rhodobacteraceae* (11.9% relative abundance), *Planococcaceae* (10.5% relative abundance) and *Xanthomonadaceae* (8.8% relative abundance) under the nitrate-reducing conditions; *Carnobacteriaceae* (17.9% relative abundance), *Geobacteraceae* (8.9% relative abundance) and *Anaerolinaceae* (8.6% relative abundance) under the iron-reducing reducing conditions; and *Pseudomonadaceae* (8.6% relative abundance), *Comamonadaceae* (9.6% relative abundance) and *Anaerolinaceae* (7.1% relative abundance) in the original inoculum. Notably, the abundant families increased significantly during cultivation under all reducing conditions, except for the phylotypes related to *Anaerolineaceae*. *Anaerolineaceae* increased significantly under the sulfate-reducing conditions and slightly under the iron-reducing conditions, but decreased obviously under the methanogenic and nitrate-reducing conditions.

PCA showed that enriched bacteria with four electron acceptors obtained significant changes from original inoculum, and bacterial communities were similar in nitrate and iron as electron acceptors (Fig. 5).

The archaeal community structures were analyzed under methanogenic and iron-reducing conditions for higher proportions. Analysis of the microbial community structures revealed that 86.2% phylotypes under the methanogenic conditions, 98.5% phylotypes under the iron-reducing conditions and 96.8% phylotypes under the original inoculum conditions could be identified to the family level. The families identified are summarized in Fig. 6: the archaeal family that was highly abundant in the methanogenic enrichment was *Methanobacteriaceae* (20.3% relative abundance); archaea families that were highly abundant in the iron-reducing enrichment were *Methanobacteriaceae* (28.2% relative abundance) and *Methanosarcinaceae* (13.5% relative abundance); and archaeal families in the original inoculum mainly consisted of *Methanobacteriaceae* (12.7% relative abundance) and *Methanosarcinaceae* (0.8% relative abundance). *Methanobacteriaceae* under the methanogenic and iron-reducing conditions obviously increased, and *Methanosarcinaceae* rose significantly under the iron-reducing conditions and almost unchanged under the methanogenic conditions.

3.3 Metabolic products of anaerobic phenanthrene biodegradation under all redox conditions

GC-MS was utilized to detect the metabolic products of anaerobic phenanthrene biodegradation, and all of the metabolic products were shown in Fig. 7. To assess whether carboxylation was a possible mechanism of phenanthrene metabolism, the 2- phenanthroic acid standard was used in this study. Mass spectral analysis of the 2-phenanthroic acid standard contained the ions m/z 294, 279, 235, 205, 177, 139, and 88. The ion m/z 294 was the molecular weight of derivatized 2-phenanthroic acid; 177 was the ion m/z for the loss of the derivatized-COO group during mass spectral fragmentation; and 2-phenanthroic acid was detected in enrichment cultures under all of the reducing conditions (Fig. S1). GC-MS analysis showed similar mass fragments to the trimethylsilyl derivative of dihydro-2-phenanthroic acid (m/z ion fragments of 90, 141, 178, 207, 237, 281 and 296 for the trimethylsilyl derivative) were detected in the sulfate-reducing, methanogenic and nitrate-reducing cultures (Fig. S2). The trimethylsilyl derivative of hexahydro-2-phenanthroic acid (m/z ion fragments of 95, 145, 183, 211, 241, 285 and 300) was also detected under all redox conditions (Fig. S3). Octahydro-2-phenanthroic acid (m/z ion fragments of 95, 147, 185, 213, 243, 287 and 302 for the trimethylsilyl derivative) and p-cresol (m/z ion fragments

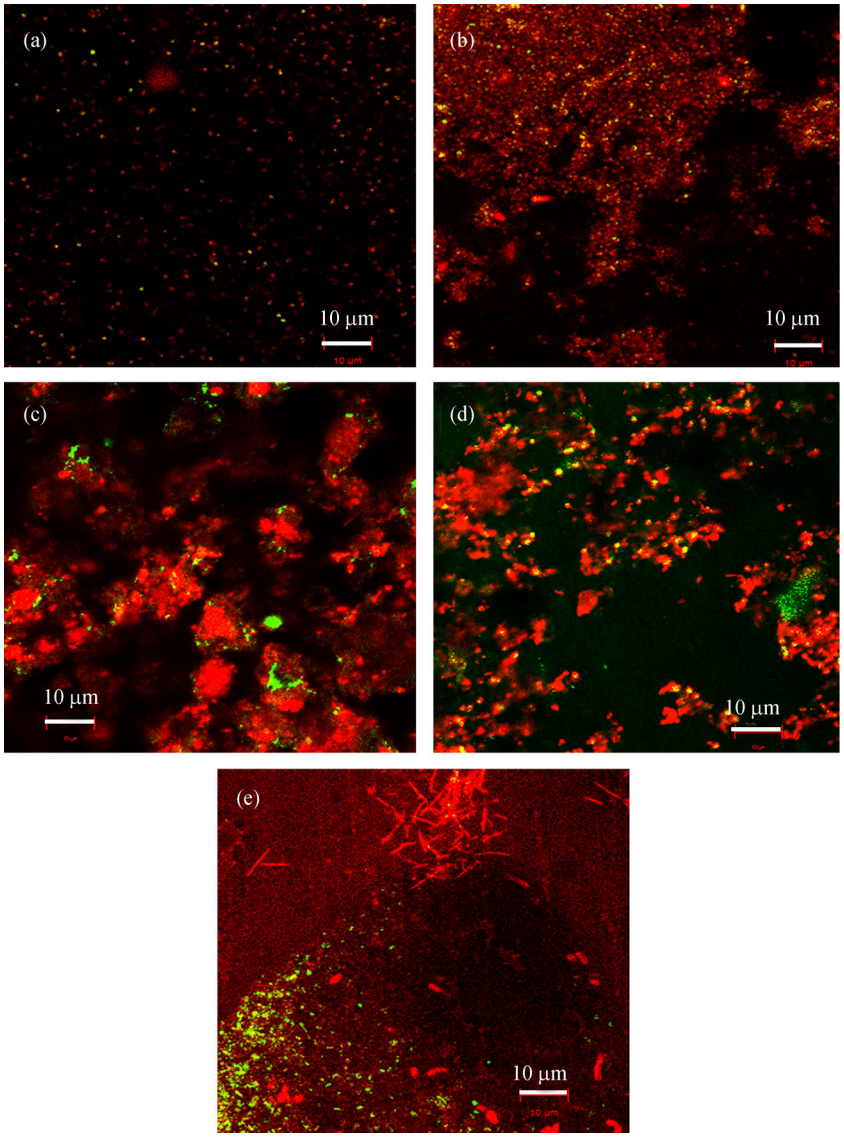


Fig. 3 FISH analysis for community composition. (a) sulfate-reducing conditions; (b) methanogenic conditions; (c) nitrate-reducing conditions; (d) iron-reducing conditions; (e) original inoculum. Red excitation represents bacteria (Cy5-labeled); green excitation represents archaea (Cy3-labeled). Bar: 10 μm.

of 77, 90, 107, 135, 165 and 180 for the trimethylsilyl derivative) were detected in the sulfate-reducing, methanogenic and nitrate-reducing cultures (Figs. S4 and S5). Cyclohex-1en-1-ol (m/z ion fragments of 69, 117, and 132 for the trimethylsilyl derivative) was detected under methanogenic, nitrate-reducing, and iron-reducing conditions (Fig. S6).

4 Discussion

The anaerobic environments are widespread on earth, and PAHs are persistent pollutants in the environment and are harmful to humans and wildlife. PAHs biodegradation is a popular research area, but researchers have mainly focused on aerobic PAHs biodegradation (Smith, 1990; Li et al., 2017). Currently, a growing number of scholars are investigating anaerobic biodegradation of PAHs. Studies on the anaerobic phenanthrene biodegradation characteristics, microbial community structures, and metabolic products under sulfate-reducing, methanogenic, nitrate-reducing, and iron-reducing conditions are necessary to guide the bioremediation of large PAHs. In this study, we provide information about the anaerobic phenanthrene biodegradation properties, communities structures, and metabolic processes.

After 244 days of enrichment, the anaerobic phenanthrene biodegradation rates tended to stabilize. The high-to-low order of anaerobic phenanthrene biodegradation rates under all reducing conditions was: nitrate-reducing

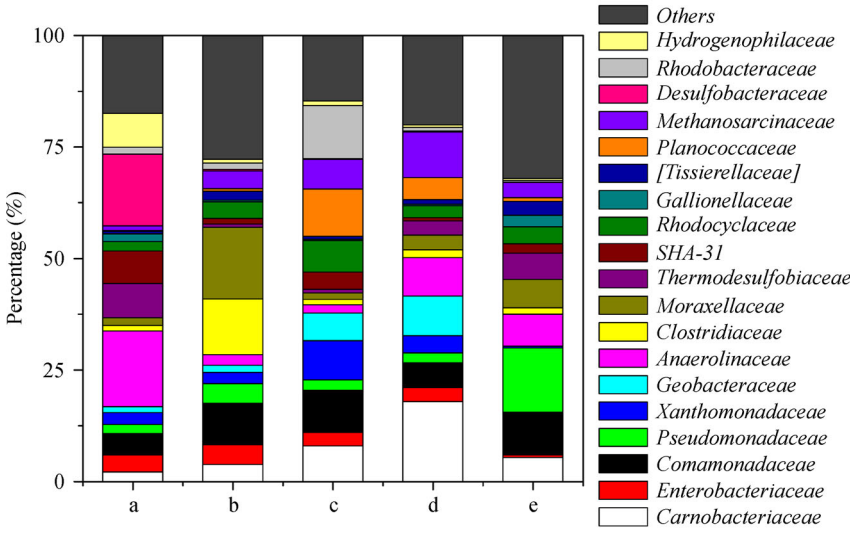


Fig. 4 Bacterial community structures. (a) sulfate-reducing conditions; (b) methanogenic conditions; (c) nitrate-reducing conditions; (d) iron-reducing conditions; (e) original inoculum.

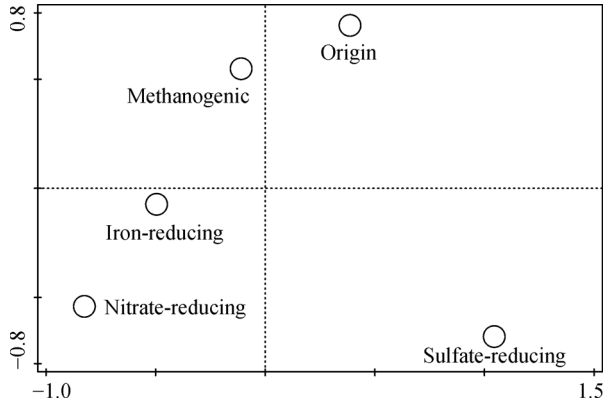


Fig. 5 Principal component analysis (PCA) of anaerobically phenanthrene-degrading bacterial community compositions.

conditions>sulfate-reducing conditions>methanogenic conditions>iron-reducing conditions, somewhat different from results in previous studies. Chang et al. indicated a high-to-low order of anaerobic phenanthrene biodegradation rates of sulfate-reducing conditions>methanogenic conditions>nitrate-reducing conditions (Chang et al., 2003). Sharak Genthner et al. obtained the order of anaerobic phenanthrene biodegradation rates of methanogenic conditions>sulfate-reducing conditions>nitrate-reducing conditions (Sharak Genthner et al., 1997). These differences may be due to different original cultures. Chang et al. utilized river sediment as the original culture (Chang et al., 2003), and Sharak Genthner et al. used creosote-contaminated sediment as the source of the inoculum (Sharak Genthner et al., 1997). According to the electron gain and loss balance in anaerobic phenanthrene biodegradation with different electron acceptors, most of the sulfate was reduced to sulfide under sulfate-reducing conditions. A small amount of nitrate was reduced to nitrite, and the remainder was converted to nitrogen under nitrate-reducing conditions, and ferric iron was all transformed to ferrous iron under the iron-reducing conditions. Ion forms conversion was similar to anaerobic naphthalene biodegradation.

The microbial proliferation of anaerobic PAHs biodegradation was indicated by measuring VSS, FISH analysis, and real-time PCR analysis (Fuchedzhieva et al., 2008; Kleemann and Meckenstock, 2011). In this study, the high-to-low order of the VSS increase was iron-reducing conditions>nitrate-reducing conditions>methanogenic conditions>sulfate-reducing conditions, which disagreed with the anaerobic phenanthrene biodegradation rates. This may be due to the different assimilation efficiencies of anaerobic phenanthrene biodegradation under various reducing conditions. Rockne and Strand showed that 57% naphthalene carbon was assimilated under nitrate-reducing conditions, but some scholars indicated that only 30%–40% naphthalene carbon was assimilated under sulfate-reducing conditions (Galushko et al., 1999; Rockne and Strand, 2001). In thermodynamics aspect, VSS increasing order should be: iron-reducing conditions>nitrate-reducing conditions>sulfate-reducing condition>methanogenic conditions. Under methanogenic conditions, biomass increasing a little more than under sulfate conditions may be caused by that some bacteria and archaea utilized inorganic carbon for growth. Our results showed that anaerobic phenanthrene biodegradation might be enriched most easily under iron-reducing conditions.

Bacteria and archaea might be involved in anaerobic PAHs biodegradation. Some communities were obtained for anaerobic single to three-cycle aromatic hydrocarbons

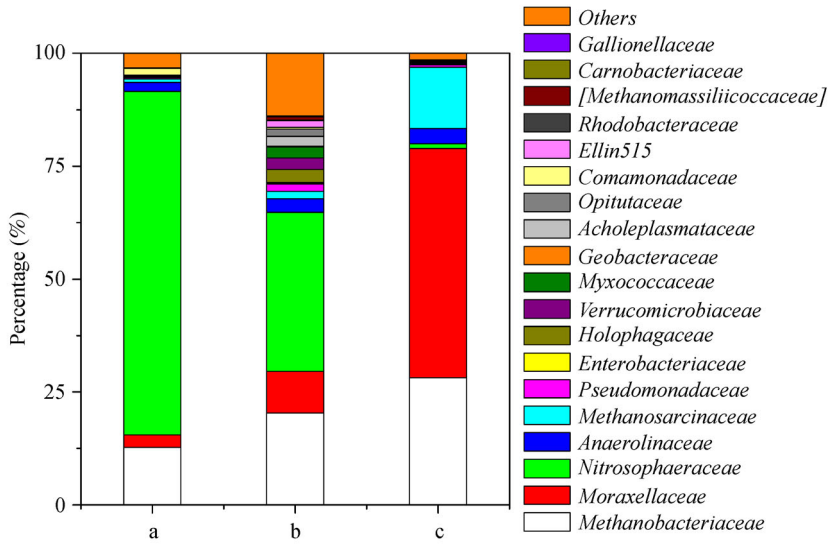


Fig. 6 Archaea community structures. (a) original inoculum; (b) methanogenic conditions; (c) iron-reducing conditions.

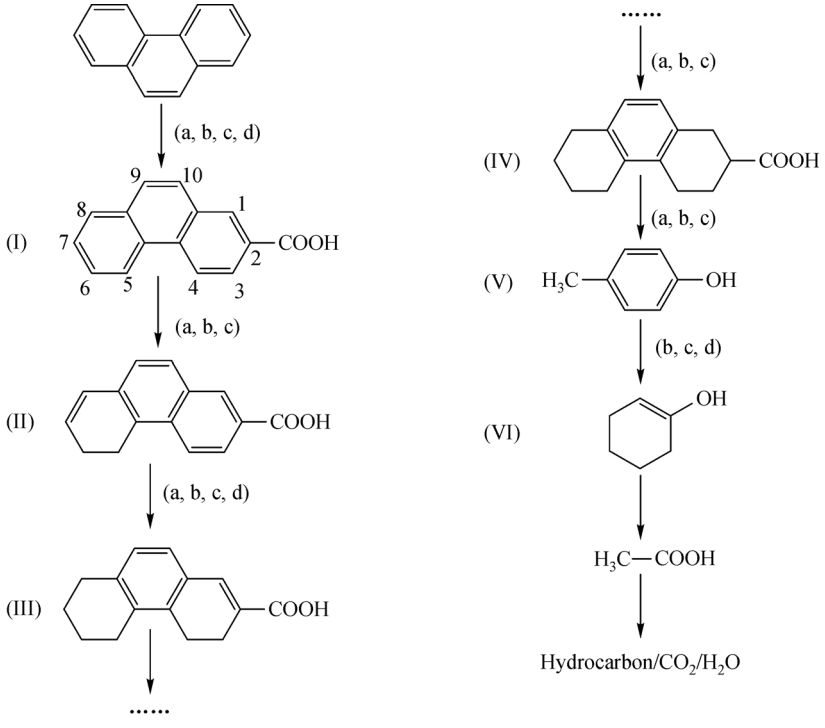


Fig. 7 Metabolic products for anaerobic phenanthrene degradation. (a) sulfate-reducing conditions; (b) methanogenic conditions; (c) nitrate-reducing conditions; (d) iron-reducing conditions. (I) 2-phenanthrene carboxylic acid, (II) dihydro-2-phenanthrene carboxylic acid, (III) hexahydro-2-phenanthrene carboxylic acid, (IV) octahydro-2-phenanthrene carboxylic acid, (V) p-cresol, (VI) cyclohex-1-en-1-ol.

biodegradation under various reducing conditions in some studies. Davidova et al. obtained a phenanthrene-degrading enrichment under the sulfate-reducing conditions, mainly belonging to the family *Desulfobacteraceae* (Davidova et al., 2007), which was similar to anaerobic naphthalene biodegradation under the sulfate-reducing conditions. Berdugo-Clavijo et al. showed that the bacterial members most closely related to the *Clostridiaceae* were the dominant bacteria in anaerobic naphthalene biodegradation under methanogenic conditions (Berdugo-Clavijo et al., 2012). Previous studies stated that the benzene-degrading *Peptococcaceae* strain combined with

a benzoate-degrading and nitrate-reducing *Azoarcus* strain (belonging to family *Rhodocyclaceae*) could catabolize benzene completely with nitrate as the terminal electron acceptor (Sharak Genthner et al., 1997; Luo et al., 2014). Aromatic carboxylic acid biodegradation and aromatic-degrading genes from the typical nitrate-reducing *beta proteobacteria Azoarcus spp.* were significantly enriched after nitrate addition, and *Dechloromonas* (belonging to family *Rhodocyclaceae*), *Pseudomonas*, *Ralstonia*, and *Sphingomonas* were found to be involved in the biodegradation of various sole-cycle aromatic compounds under nitrate-reducing conditions (Xu et al., 2015). Kleemann and Meckenstock obtained the enriched culture N49 (mainly composed of bacteria belong to family *Peptococcaceae*), which was able to biodegradation naphthalene under iron-reducing conditions (Kleemann and Meckenstock, 2011), the main bacteria was similar with benzene biodegradation under iron-reducing conditions. Müller et al. showed that *Geobacteraceae* might participate in anaerobic biodegradation of naphthalene under iron-reducing conditions (Müller et al., 2017). Our results clearly showed that the enrichments community structures mainly included *Desulfobacteraceae*, *Anaerolinaceae,* and *Thermodesulfobiaceae* under sulfate-reducing conditions; *Moraxellaceae*, *Clostridiaceae,* and *Comamonadaceae* under methanogenic conditions; *Rhodobacteraceae*, *Planococcaceae,* and *Xanthomonadaceae* under nitrate-reducing conditions; and *Geobacteraceae*, *Carnobacteriaceae,* and *Anaerolinaceae* under iron-reducing conditions. Some of the dominant bacteria families in our study were analogous to the studies mentioned above under corresponding reducing conditions. *Geobacteraceae*, which plays an important role in the interspecies electron transfer, increased in various redox conditions, and iron-reducing conditions promoted hydrogenotrophic methanogens, which has been reported that extracellular electron transfer plays an important role to the interspecies metabolic pathway. Bacterial communities were similar, taking nitrate and iron as electron acceptors indicated that many same bacteria families might survive in nitrate-reducing conditions and iron-reducing conditions or that some bacteria owned both nitrate-reducing and iron-reducing activities. Zhang et al. showed that the phylum Euryarchaeota was the predominant archaeal group, represented by the genera *Methanosarcina*, *Methanobacterium* and *Thermogymnomonas,* and indicated that it might have links to phenanthrene biodegradation under methanogenic conditions (Zhang et al., 2012). Our studies found that archaea affiliated to the *Methanobacteriaceae* family were enriched under methanogenic conditions, which verified that *Methanobacteriaceae* family might be involved in phenanthrene biodegradation under methanogenic conditions. Our first finding was that *Methanosarcinaceae* and *Methanobacteriaceae* were enriched when phenanthrene was used as a carbon source under iron-reducing conditions.

Carboxylation or methylation is regarded as the initial step of anaerobic PAH biodegradation, and the anaerobic phenanthrene biodegradation metabolic processes were only identified under sulfate-reducing conditions for obtaining of three reasonable enrichments (Zhang and Young, 1997; Davidova et al., 2007; Himmelberg et al., 2018). The anaerobic metabolic products of naphthalene are widely reported due to the isolation of pure cultures of NaphS2, NaphS, and the core microbiome N47. Carboxylation is the key step in naphthalene biodegradation under sulfate-reducing conditions (Mouttaki et al., 2012). Previous studies identified tetrahydro-2-naphthoic acid and octahydro-2-naphthoic as reduced derivatives of 2-naphthoic acid (Zhang et al., 2000; Annweiler et al., 2002) and the gene sequence of naphthoyl-CoA reductase *(Ncr)* for anaerobic naphthalene biodegradation under sulfate-reducing conditions was also proposed (Morris et al., 2014). Further biodegradation occurred via a cyclohexane ring structure rather than mono-aromatic compounds (Annweiler et al., 2002). Carboxylation, ring reduction, and ring cleavage were important steps in anaerobic naphthalene biodegradation. As for anaerobic phenanthrene biodegradation, direct carboxylation was proposed as initial reaction step, and ring reduction steps had been recently identified in one sulfate-reducing enrichments, based on observations of 2-phenanthroic acid being converted to decahydro-2-phenanthroic acid (Himmelberg et al., 2018). However, description of anaerobic phenanthrene biodegradation processes in the downstream and with other electron acceptors except sulfate is still in their infancy.

Similarly, our study indicated that ring reduction, ring cleavage, and carboxylation were also key steps in anaerobic phenanthrene biodegradation under four redox conditions. Carboxylation was the initial step of anaerobic phenanthrene biodegradation under all redox conditions. Dihydro-2-phenanthroic acid, hexahydro-2-phenanthroic acid, octahydro-2-phenanthroic acid, p-cresol, and cyclohex-1-en-1-ol could be metabolic products of anaerobic phenanthrene biodegradation. Substituted benzene series, which had not been observed in anaerobic naphthalene degradation, were identified as intermediate products of anaerobic phenanthrene degradation. A pure culture or core microbiome isolated for anaerobic phenanthrene biodegradation under various conditions, and functional genes and catalytic enzymes identification for anaerobic phenanthrene biodegradation needed to be done in the future studies.

5 Conclusions

Anaerobic phenanthrene biodegradation, including degradation rate, ion conversion, biological growth, and metabolic processes was demonstrated under sulfate-reducing, methanogenic, nitrate-reducing, and

iron-reducing conditions. The enriched cultures under all of the redox conditions were obtained from the same initial inoculum. Bacteria populations under all of the redox conditions and archaea populations under the methanogenic and iron-reducing conditions were demonstrated, and the bacterial communities were similar under nitrate-reducing and iron-reducing conditions. Relatively intact pathways of anaerobic phenanthrene biodegradation under methanogenic, nitrate-reducing and iron-reducing conditions were upregulated. This study showed different electron acceptors induced different communities, but parallel metabolic pathway. Carboxylation was confirmed to be the key anaerobic phenanthrene biodegradation step under all redox conditions. Ring reduction followed by ring cleavage proved to be important in anaerobic phenanthrene biodegradation after carboxylation.

Acknowledgements This study was funded by the National Natural Science Foundations of China (Grant Nos. 41573065 and 41773082), the Key Project of Natural Science Foundation of China (No. 21337001) and the Mega-projects of Science Research for Water Environment Improvement (No. 2017ZX07202002).

Electronic Supplementary Material Supplementary material is available in the online version of this article at https://doi.org/10.1007/s11783-019-1164-x and is accessible for authorized users.

References

Amann R, Fuchs B M (2008). Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques. Nature Reviews. Microbiology, 6(5): 339–348

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

APHA (1998). Standard Methods for the Examination of Water and Wastewater. Baltimore MD: American Public Health Association

Bauer J E, Capone D G (1985). Degradation and mineralization of the polycyclic aromatic hydrocarbons anthracene and naphthalene in intertidal marine sediments. Applied and Environmental Microbiology, 50(1): 81–90

Berdugo-Clavijo C, Dong X, Soh J, Sensen C W, Gieg L M (2012). Methanogenic biodegradation of two-ringed polycyclic aromatic hydrocarbons. FEMS Microbiology Ecology, 81(1): 124–133

Chang B V, Chang S W, Yuan S Y (2003). Anaerobic degradation of polycyclic aromatic hydrocarbons in sludge. Advances in Environmental Research, 7(3): 623–628

Coates J D, Anderson R T, Woodward J C, Phillips E J P, Lovley D R (1996). Anaerobic hydrocarbon degradation in petroleum-contaminated harbor sediments under sulfate-reducing and artificially imposed iron-reducing conditions. Environmental Science & Technology, 30(9): 2784–2789

Davidova I A, Gieg L M, Duncan K E, Suflita J M (2007). Anaerobic phenanthrene mineralization by a carboxylating sulfate-reducing bacterial enrichment. The ISME journal, 1(5): 436–442

Fang T, Pan R, Jiang J, He F, Wang H (2016). Effect of salinity on community structure and naphthalene dioxygenase gene diversity of a halophilic bacterial consortium. Frontiers of Environmental Science & Engineering, 10(6): 16

Feng Z J, Zhu L Z (2018). Sorption of phenanthrene to biochar modified by base. Frontiers of Environmental Science & Engineering, 12 (2): 1

Fuchedzhieva N, Karakashev D, Angelidaki I (2008). Anaerobic biodegradation of fluoranthene under methanogenic conditions in presence of surface-active compounds. Journal of Hazardous Materials, 153(1–2): 123–127

Galushko A, Minz D, Schink B, Widdel F (1999). Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium. Environmental Microbiology, 1(5): 415–420

Hambrick G A, Delaune R D, Patrick W H (1980). Effect of estuarine sediment pH and oxidation-reduction potential on microbial hydrocarbon degradation. Applied and Environmental Microbiology, 40(2): 365–369

Himmelberg A M, Brüls T, Farmani Z, Weyrauch P, Barthel G, Schrader W, Meckenstock R U (2018). Anaerobic degradation of phenanthrene by a sulfate-reducing enrichment culture. Environmental Microbiology, 20(10): 3589–3600

Kleemann R, Meckenstock R U (2011). Anaerobic naphthalene degradation by Gram-positive, iron-reducing bacteria. FEMS Microbiology Ecology, 78(3): 488–496

Kümmel S, Herbst F A, Bahr A, Duarte M, Pieper D H, Jehmlich N, Seifert J, von Bergen M, Bombach P, Richnow H H, Vogt C (2015). Anaerobic naphthalene degradation by sulfate-reducing *Desulfobacteraceae* from various anoxic aquifers. FEMS Microbiology Ecology, 91(3): 1–12: fiv006

Langenhoff A A M, Zehnder A J B, Schraa G (1996). Behaviour of toluene, benzene and naphthalene under anaerobic conditions in sediment columns. Biodegradation, 7(3): 267–274

Li J, Luo C, Song M, Dai Q, Jiang L, Zhang D, Zhang G (2017). Biodegradation of phenanthrene in polycyclic aromatic hydrocarbon-contaminated wastewater revealed by coupling cultivation-dependent and-independent approaches. Environmental Science & Technology, 51(6): 3391–3401

Luo F, Gitiafroz R, Devine C E, Gong Y, Hug L A, Raskin L, Edwards E A (2014). Metatranscriptome of an anaerobic benzene-degrading, nitrate-reducing enrichment culture reveals involvement of carboxylation in benzene ring activation. Applied and Environmental Microbiology, 80(14): 4095–4107

Luo J, Zhang J, Tan X, McDougald D, Zhuang G, Fane A G, Kjelleberg S, Cohen Y, Rice S A (2015). Characterization of the archaeal community fouling a membrane bioreactor. Journal of Environmental Sciences-China, 29: 115–123

Martirani-Von Abercron S M, Pacheco D, Benito-Santano P, Marín P, Marqués S (2016). Polycyclic aromatic hydrocarbon-induced changes in bacterial community structure under anoxic nitrate reducing conditions. Frontiers in Microbiology, 7: 1–16

Meckenstock R U, Annweiler E, Michaelis W, Richnow H H, Schink B (2000). Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. Applied and Environmental Microbiology, 66(7):

2743–2747

Mihelcic J R, Luthy R G (1988). Degradation of polycyclic aromatic hydrocarbon compounds under various redox conditions in soil-water systems. Applied and Environmental Microbiology, 54(5): 1182–1187

Morris B E, Gissibl A, Kümmel S, Richnow H H, Boll M (2014). A PCR-based assay for the detection of anaerobic naphthalene degradation. FEMS Microbiology Letters, 354(1): 55–59

Mouttaki H, Johannes J, Meckenstock R U (2012). Identification of naphthalene carboxylase as a prototype for the anaerobic activation of non-substituted aromatic hydrocarbons. Environmental Microbiology, 14(10): 2770–2774

Müller J B, Ramos D T, Larose C, Fernandes M, Lazzarin H S, Vogel T M, Corseuil H X (2017). Combined iron and sulfate reduction biostimulation as a novel approach to enhance BTEX and PAH source-zone biodegradation in biodiesel blend-contaminated groundwater. Journal of Hazardous Materials, 326: 229–236

Musat F, Galushko A, Jacob J, Widdel F, Kube M, Reinhardt R, Wilkes H, Schink B, Rabus R (2009). Anaerobic degradation of naphthalene and 2-methylnaphthalene by strains of marine sulfate-reducing bacteria. Environmental Microbiology, 11(1): 209–219

Obi C C, Adebusoye S A, Amund O O, Ugoji E O, Ilori M O, Hedman C J, Hickey W J (2017). Structural dynamics of microbial communities in polycyclic aromatic hydrocarbon-contaminated tropical estuarine sediments undergoing simulated aerobic biotreatment. Applied Microbiology and Biotechnology, 101(10): 4299–4314

Rockne K J, Strand S E (2001). Anaerobic biodegradation of naphthalene, phenanthrene, and biphenyl by a denitrifying enrichment culture. Water Research, 35(1): 291–299

Safinowski M, Meckenstock R U (2006). Methylation is the initial reaction in anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. Environmental Microbiology, 8(2): 347–352

Sharak Genthner B R, Townsend G T, Lantz S E, Mueller J G (1997). Persistence of polycyclic aromatic hydrocarbon components of creosote under anaerobic enrichment conditions. Archives of Environmental Contamination and Toxicology, 32(1): 99–105

Smith M R (1990). The biodegradation of aromatic hydrocarbons by bacteria. Biodegradation, 1(2–3): 191–206

Tor J M, Lovley D R (2001). Anaerobic degradation of aromatic compounds coupled to Fe(III) reduction by Ferroglobus placidus. Environmental Microbiology, 3(4): 281–287

Trably E, Patureau D, Delgenes J P (2003). Enhancement of polycyclic aromatic hydrocarbons removal during anaerobic treatment of urban sludge. Water Science and Technology, 48(4): 53–60

Weyrauch P, Zaytsev A V, Stephan S, Kocks L, Schmitz O J, Golding B T, Meckenstock R U (2017). Conversion of cis-2-carboxycyclohexylacetyl-CoA in the downstream pathway of anaerobic naphthalene degradation. Environmental Microbiology, 19(7): 2819–2830

Xu M, He Z, Zhang Q, Liu J, Guo J, Sun G, Zhou J (2015). Responses of aromatic-degrading microbial communities to elevated nitrate in sediments. Environmental Science & Technology, 49(20): 12422–12431

Yarza P, Yilmaz P, Pruesse E, Glöckner F O, Ludwig W, Schleifer K H, Whitman W B, Euzéby J, Amann R, Rosselló-Móra R (2014). Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. Nature Reviews. Microbiology, 12(9): 635–645

Ye Q H, Wang C Y, Wang Y, Wang H (2018). Characterization of a phenanthrene-degrading methanogenic community. Frontiers of Environmental Science & Engineering, 12 (5): 4

Yuan S Y, Chang B V (2007). Anaerobic degradation of five polycyclic aromatic hydrocarbons from river sediment in Taiwan. Journal of Environmental Science and Health. Part. B, Pesticides, Food Contaminants, and Agricultural Wastes, 42(1): 63–69

Zhang S Y, Wang Q F, Xie S G (2012). Molecular characterization of phenanthrene-degrading methanogenic communities in leachate-contaminated aquifer sediment. International Journal of Environmental Science and Technology, 9(4): 705–712

Zhang X, Sullivan E R, Young L Y (2000). Evidence for aromatic ring reduction in the biodegradation pathway of carboxylated naphthalene by a sulfate reducing consortium. Biodegradation, 11(2/3): 117–124

Zhang X, Young L Y (1997). Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. Applied and Environmental Microbiology, 63(12): 4759–4764