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RESEARCH ARTICLE

Characterization of a phenanthrene-degrading methanogenic community

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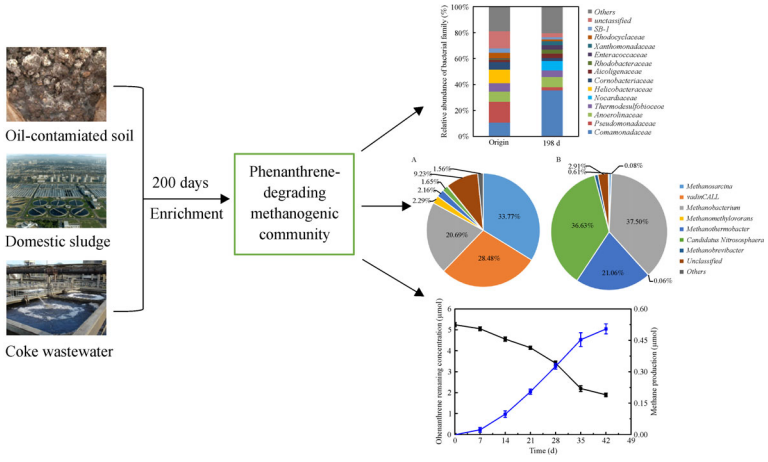
HIGHLIGHTS

- The mixed samples of contaminated soil, sludge and coke wastewater showed great phenanthrene methanogenic degradation potential.
- *Comamonadaceae*, *Nocardiaceae* and *Methanobacterium* were dominant members.
- Hexane, hexadecane and benzene could enhance phenanthrene degradation.

GRAPHIC ABSTRACT



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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) often occur in oil-contaminated soil, coke wastewater and domestic sludge; however, associated PAH degraders in these environments are not clear. Here we evaluated phenanthrene degradation potential in the mixed samples of above environments, and obtained a methanogenic community with different microbial profile compared to those from sediments. Phenanthrene was efficiently degraded (1.26 mg/L/d) and nonstoichiometric amount of methane was produced simultaneously. 16S rRNA gene sequencing demonstrated that bacterial populations were mainly associated with *Comamonadaceae Nocardiaceae* and *Thermodesulfobiaceae*, and that methanogenic archaea groups were dominated by *Methanobacterium* and *Methanothermobacter*. Substances such as hexane, hexadecane, benzene and glucose showed the most positive effects on phenanthrene degradation. Substrate utilization tests indicated that this culture could not utilize other PAHs. These analyses could offer us some suggestions on the putative phenanthrene-degrading microbes in such environments, and might help us develop strategies for the removal of PAHs from contaminated soil and sludge.

1 Introduction

The study of polycyclic aromatic hydrocarbons (PAHs) has aroused much attention due to their carcinogenic, teratogenic and mutagenic effects on human beings and wildlife health through food chain. Some of them even have neurovirulence. Early in 1976, 16 PAHs were included in the list of priority persistent pollutants (Keith and Telliard, 1979; Bengtsson and Zerhouni, 2003). Thus, it is

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important to effectively remove them from our environment.

Microbial biodegradation has been considered to be an effective and green approach to their removal from our environment. During the past decades, numerous aerobic degraders of PAHs have been isolated and related degradation mechanisms have been fully investigated (Chen and Aitken, 1999; Haritash and Kaushik, 2009). In late 20th century, some PAHs were found to be degraded in the absence of oxygen. For example, several enriched cultures initiated with harbor sediments under sulfate-reducing condition were capable of utilizing PAHs including naphthalene (Zhang and Young, 1997; Safinowski and Meckenstock, 2006), 2-methylnaphthalene (Sullivan et al., 2001) and phenanthrene (Zhang and Young, 1997; Davidova et al., 2007). To date, some pure naphthalene/2-methylnaphthalene-degrading strains like NaphS2 (Galushko et al., 1999), NaphS3 and NaphS6 (Musat et al., 2009) under sulfate-reducing condition have been isolated. To my knowledge, nearly all enrichment cultures and pure strains obtained under sulfate-reducing condition were closely affiliated with *Desulfobacteraceae* (Kümmel et al., 2015).

More recently, PAHs were also reported to be degraded in harbor sediment under anaerobic methanogenic condition (Chang et al., 2005). In real surroundings, methanogenic condition can be formed by using carbon dioxide as electron acceptors due to the depletion of oxygen, sulfate, nitrate and metal ions. However, PAHs degradation potentials under anaerobic methanogenic condition in many typical surroundings were unreported. Besides, PAHs degradation under anaerobic methanogenic condition consists of several main steps such as hydrolysis, acidification and methanogenesis, and is often carried out by different groups of microorganisms. Thus, compared to sulfate-reducing condition, microbes responsible for PAHs degradation under anaerobic methanogenic condition tend to be more diverse and complex. However, species diversity of PAHs degraders under this anaerobic condition was scarcely explored. Chang investigated PAHs degradation potentials in Baltimore harbor sediment and characterized the community structures of two cultures developed from this sediment under anaerobic methanogenic condition. Naphthalene and phenanthrene could effectively be utilized by each culture, respectively. The phylogenetic analysis indicated that the dominant microbial groups were affiliated with *Firmicutes* and *Methanococci* in naphthalene-degrading culture, while phenanthrene-degrading culture was mainly related to *Proteobacteria* and *Methanomicrobia* (Chang et al., 2005). In addition to harbor sediment, PAHs degradation potentials and associated microbes were also investigated using aquifer sediment as inoculum source. For example, Zhang found that phenanthrene could be effectively degraded in leachate-contaminated aquifer sediment and that the main genera of bacteria and archaea were affiliated with *Citrobacter* and *Methanobacterium*, respectively (Zhang et al., 2012b). Anthracene was also degraded in such aquifer sediments and different microorganisms (*Methylibium* and *Legionella*) might be responsible for anthracene removal (Wan et al., 2012; Zhang et al., 2012a). Apart from sediments, PAHs also exist in some other surroundings such as oil-contaminated soil (Gómez et al., 2004), coke wastewater (Chang et al. 2003) and domestic sludge (Pérez et al., 2001). The PAH degradation potential and associated degraders in these surroundings might be different from those in sediments. However, such information about PAHs degradation potential and degraders was unavailable.

In the present study, we collected samples from typical anaerobic environments such as oil-contaminated soil, anaerobic domestic sludge and coke wastewater, and mixed them. We explored the abilities of phenanthrene degradation in the mixed environment samples and obtained a phenanthrene-degrading methanogenic community. Then the diversity of anaerobic phenanthrene degraders was characterized through Illumina sequencing technology. Finally, the effects of different substances on phenanthrene degradation and substrate utilization diversity of this enrichment culture were investigated. These analyses could provide an important theoretical support for evaluation of PAHs fate in anaerobic environments and for PAHs remediation under anaerobic conditions.

2 Materials and methods

2.1 Inoculum source

The soil samples were collected from the subsurface petroleum-contaminated soil of Shengli Oil field in Shangdong Province. The coke wastewater was from a wastewater treatment plant of a steel company in Hebei Province, and the anaerobic domestic sludge was obtained from Gaobeidian wastewater treatment plant in Beijing. Samples were immediately brought to our laboratory and treated with nitrogen gas, then they were stored at 4°C before use.

2.2 Development of the methanogenic phenanthrene-degrading consortium

The enrichment of phenanthrene-degrading consortium under anaerobic methanogenic condition was conducted in 1 L conical flask (1 L working volume) placed in a six-header magnetic stirring apparatus (temperature: 32°C, speed: 30). 20–25 mg/L phenanthrene-acetone solution was provided as the sole carbon source. After the acetone volatized from the flask, the mixed environment samples (10 g petroleum-contaminated soil, 10 mL domestic sludge and 10 mL coke wastewater) were added. The culture medium, trace elements and vitamin nutrients described in

our previous work (Ye et al., 2018) were immediately provided to make up the remaining volume. 20 mM sodium bicarbonate was supplemented as electron acceptors. After bubbling the flask with high purify nitrogen for 20mins, pH was adjusted to 7.3–7.5 and 10 mg sodium sulphide was added to remove the remaining oxygen in the medium. Finally, the flask was sealed with a rubber stopper with three predrilled holes (one is for collecting gas, one is for temperature probe and one is for taking samples) (Ye et al., 2018). At regular time, samples were taken to measure the remaining phenanthrene. When most phenanthrene was degraded, new nutrient solution was provided and phenanthrene was supplemented to 20−25 mg/L. A phenathrene-degrading consortium under anaerobic methanogenic condition was obtained after the 12th phase.

2.3 Analytic methods

Phenanthrene in the sample was extracted with 5-time volumes methylene dichloride, then the extracted liquor was purged into liquid vial by passing through 0.22 μm organic filters, and the concentration was determined with High Performance Liquid Chromatography (HPLC-20AD) followed the method of Fang (Fang et al., 2016). Methane production was determined through Gas Chromatography (GC-2014) equipped with thermal conductivity detector and methane conversion oven, the measurement condition were as follow: high purify nitrogen as carrier gas, flow rate at 30 mL/min, methane conversion oven at 300°C, chromatographic column and detector at 100°C. Methane amounts were calculated on the basis of standard curves containing known methane concentrations (Berdugo-Clavijo et al., 2012). OD was measured with ultraviolet spectrophotometer under the wavelength of 600 nm.

2.4 DNA extraction and 16S rRNA gene sequencing

The initial mixed samples were stored at −80°C before the total DNA was extracted. At day 198, samples were taken from the well-mixed flask for three consecutive times. The total DNA of the initial mixed sample and the enrichment culture were extracted using Fast DNA® SPIN Kit for Soil (MP Biomedicals, USA) following the manufacturer's instructions. The qualities of the DNA samples were examined through NanoDrop 2000 Spectrophotometer (Thermo Fisher, USA) and gel electrophoresis (Powerpac basic, Bio-Rad), and the qualified DNA samples were mixed respectively. Finally, the mixed DNA samples were sent to SinoBioCore Biological Technology Co. Ltd for Illumina sequencing. The bacterial DNA was amplified using the primer pairs 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) to target the V4 region of the 16S rRNA gene (Yarza et al., 2014). The archaeal DNA was amplified using the primers U519F (5′-CAGYMGCCRCGGKAAHACC-3′) and U806R (5′-GGACTACNSGGGTMTCTAAT-3′) (Luo et al., 2015). PCR amplification was conducted three times, and the purified products using the Axy Prep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) (Kong et al., 2018) were mixed well. The products from different samples were mixed at equal ratios for sequencing using the Illumina HISEQ 2500 platform and PE250 was chosen as the sequencing mode. Paired-end reads were merged into single sequences using PANDASEQ, then the low-quality sequences were removed (Zhuang et al., 2015). The high-quality sequences were analyzed QIIME software. Operational taxonomic units (OTUs) at 97% similarity was used to characterize the community diversity using UPARSE software package and the Genbank database was used for the taxonomic assignment.

2.5 Relationship between phenanthrene degradation and methane production

To evaluate the capabilities of phenanthrene degradation and methane production in this enrichment culture, a set of batch experiment was conducted. 200 mL anaerobic serum bottles were used, each containing 8 mL anaerobic phenanthrene-degrading consortium (OD = 1.5) and 32 mL nutrient medium described previously, spiked with 25 mg/L phenanthrene. After anaerobic treatment and pH adjustment (pH: 7.3−7.5) following the method of our previous study (Yin et al., 2017), all bottles were incubated in a horizontal shake (165 rpm and 32°C). At regular time, methane production was measured before triplicate bottles was extracted to determine the remaining phenanthrene.

2.6 Effects of different substances on phenanthrene degradation

Eight mL anaerobic phenanthrene-degrading consortium and 32 mL nutrient medium were added to 200 mL anaerobic serum bottles, and 25 mg/L phenanthrene was supplemented, then different substances were added. All bottles were placed in horizontal incubator after the anaerobic treatment following the method in Section 2.2. The Types and dosages of different substances can be found in Table S1 (See it in Supplementary material). At day 45, triplicate bottles was used for residual phenanthrene determination. All data were fitted to first-order kinetics, $S = S_0 \exp(-kt)$, where S_0 is the initial concentration, S the substrate concentration, t the time period, and k the degradation rate constant.

2.7 Substrate utilization tests

All substrate utilization tests were conducted in 200 ml anaerobic serum bottles. Each bottle was spiked with 25 mg/L different substrates (benzene, naphthalene, 2-methylnaphthalene, phenanthrene, phenanthrenecarboxylic acid, anthracene, fluoranthene and pyrene),

supplementing with 8 mL phenanthrene-degrading methanogenic culture (OD = 1.5) and 32 mL nutrient medium described previously. Anaerobic treatment and pH adjustment followed the method described in Section 2.4. After incubation for 45 days, the whole bottle was extracted to determine the remaining concentration of the provided substrate.

3 Results and discussion

3.1 Phenanthrene degradation potential and rates

Degradation data at different enrichment stages were fitted to zero-order kinetics. Figure 1 shows phenanthrene degradation rates during different enrichment phases. As shown in Fig. 1, phenanthrene degradation rates were 4–5 mg/L/d during the initial two enrichment phases (phase 1–2), greater than the degradation rate in the 12th phase. The adsorption materials in initial samples, might adsorb phenanthrene, but our laboratory experiment showed that phenanthrene extraction recovery rate could be 85%; Also, the adsorption materials might not be utilized by microbes and exist in the samples, thus we could say that most phenanthrene was degraded by microbes. The initial high degradation rates might result from the initial presence of some organic matters in mixed samples. For example, some hydrocarbons in the petroleum-contaminated soil could be served as cometabolism substrates, and the polysaccharide and protein in domestic sludge could be simultaneously utilized by microorganisms, thereby enhancing the growth of some degrading microbes. A previous study showed that the addition of small molecule substances (acetate, lactate and pyruvate) and municipal sewage or oil refinery sludge greatly enhanced PAH degradation in soil (Chang et al., 2002). When these organic matters were gradually utilized, the degradation rate drastically dropped (phase 2–4). Then it slowly dropped to the lowest point (1.06 mg/L/d at day 80) when microbes could merely utilize phenanthrene as carbon source. Afterwards, phenanthrene degradation rate experienced a slow growth stage (phase 6–9) as phenanthrene degraders grew and further enriched. Finally, the degradation rate remained stable at 1.26–1.29 mg/L/d (phase 10–12). It was noticeably larger than the degradation rate in enrichment culture initiated with harbor sediment (0.7–0.8 mg/L/d) (Chang et al., 2006), which might because the mixed environment samples provided much diverse phenanthrene degraders. Thus, phenanthrene degradation potential in the mixed environment samples was noticeable and an effective phenanthrene-degrading culture was obtained under anaerobic methanogenic condition.

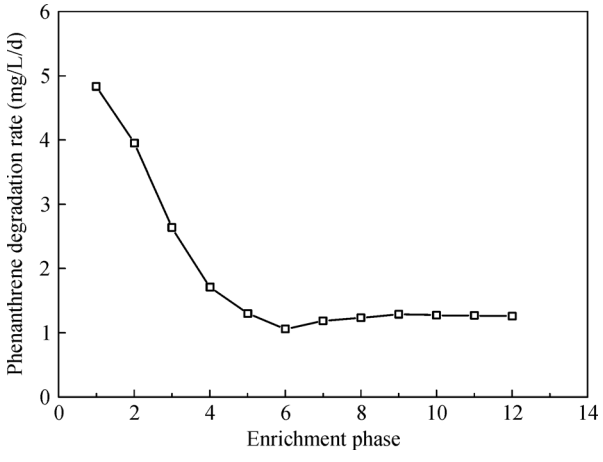

Fig. 1 Phenanthrene degradation rates of zero-order reaction in big flask during different enrichment phases; (phase 1: day 0–5; phase 2: day 7–13; phase 3: day 15−24; phase 4: day 25−39; phase 5: day 45−61; phase 6: day 66−80; phase 7: day 81−101; phase 8: day 105−121; phase 9: day 126−136; phase 10: day 137−155; phase 11: day 158−176; phase 12: day 180−198).

3.2 Bacteria diversity

The numbers of high-quality sequences of initial samples and of the enrichment samples (Table S2) were sufficient. Bacterial community structures of initial samples and the enrichment culture were presented in Fig. 2. As shown in Fig. 2a, the dominant bacteria members at class level in the initial samples were *Betaproteobacteria* (18.08%), *Gammaproteobacteria* (17.59%), *Clostridia* (12.30%), *Bacteroidia* (14.41%), *Epsilonproteobacteria* (10.63%) and *Anaerolineae* (8.97%). After enrichment for 198 days, the major ones were composed of *Betaproteobacteria* (42.12%), *Gammaproteobacteria* (13.63%), *Anaerolineae* (11.20%), *Actinobacteria* (7.73%) and *Clostridia* (7.34%). We can see that the relative abundance of *Betaproteobacteria* and *Actinobacteria* increased by 24.04% and 7.33%, respectively, while the relative abundance of *Epsilonproteobacteria* decreased by 10.62%. It is reasonable to infer that members affiliated with *Betaproteobacteria* and *Actinobacteria* might play an important role in phenanthrene degradation. In similar studies, significant different bacterial community were acquired. Zhang reported that all bacteria members were affiliated with *Gammaproteobacteria* in the phenanthrene-degrading methanogenic culture initiated from leachate-contaminated aquifer sediment (Zhang et al., 2012b). RFLP analysis for the phenanthrene-degrading culture developed from harbor sediment demonstrated that most bacteria members were associated with *Firmicutes* rather than *Proteobacteria* (3.60%) (Chang et al., 2005).

To further reveal the bacterial diversity, the results were analyzed at the lower taxonomic unit, family level. It is clear that the bacterial composition has experienced a vast change compared with the initial microbial diversity. As shown in Fig. 2b, the relative abundances of *Comamonadaceae* and *Nocardiaceae* increased by about 24.61% and 7.42%, while those of *Pseudomonadaceae* and *Helico-*

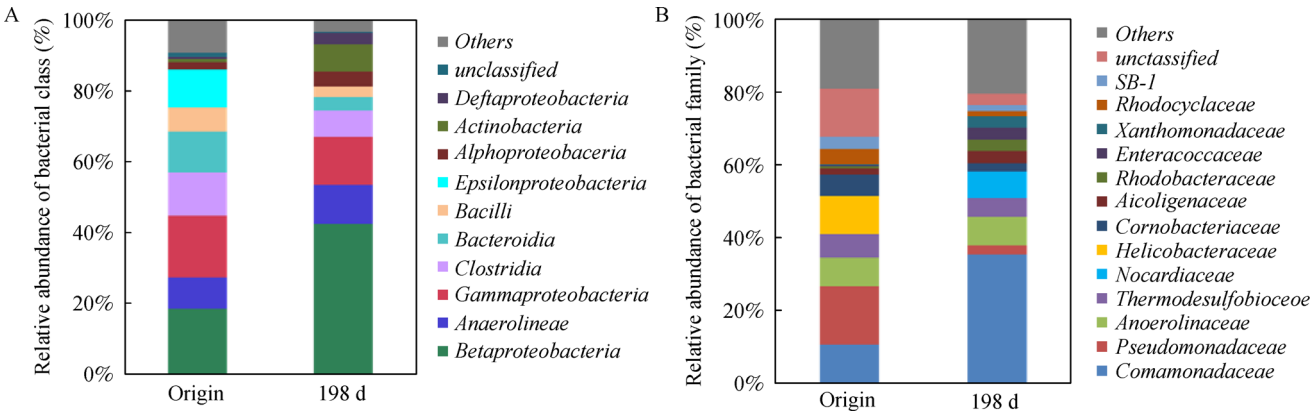

Fig. 2 Bacterial members of the initial sample and of the methanogenic enrichment culture; (A) Bacterial groups at class level; (B) Bacterial groups at family level.

bacteraceae decreased from 15.97% to 2.70%, and from 10.50% to 0.01%, respectively. The minor bacterial members in the enrichment culture also included *Thermodesulfobiaceae* (4.83%), *Alcaligenaceae* (3.46%), *Enterococcaceae* (3.39%) and *Rhodobacteraceae* (3.11%). The member *Comamonadaceae* usually occurred in hydrocarbons-contaminated soils. For example, Salvador reported that *Comamonadaceae* might play a role in biodegrading benzo(a)anthracene and chrysene due to its mere presence in the spiked slurry (Lladó et al., 2009). Clone library results showed that approximately 20% clones retrieved from bacterial biofilm of calcinated organobentonite which degraded phenanthrene belonged to family *Comamonadaceae*, and the author also found that 20% dioxygenase genes matched to those of *Comamonas* and *Mycobacterium* (Huang et al., 2013). Besides, some *Comamonas* species have been linked to degrade naphthalene and phenanthrene (Goyal and Zylstra, 1996; Tøndervik et al., 2012). Nocardiaceae could degrade hydrocarbons (e.g. petroleum distillates) and have been proposed as bioremediation agents for environmental spills (Aislabie et al., 1998). A previous study showed that *Nocardiaceae* was present in the beach sample took after heavy fuel-oil spill for one year (Jiménez et al., 2007). The other less abundant groups like *Thermodesulfobiaceae* (Liang et al., 2015), *Rhodobacteraceae* (Pinyakong et al., 2012), *Alcaligenaceae* (Canul-Chan et al., 2017) were commonly reported to degrade hydrocarbons/PAHs. Thus, different from sediment-initiated phenanthrene-degrading methanogenic community in which dominant bacteria groups were affiliated with *Enterobacteriaceae* and *Pseudomonadaceae* (Zhang et al., 2012b), the potential phenanthrene degraders in our culture were *Comamonadaceae*, *Nocardiaceae*, *Thermodesulfobiaceae* and *Alcaligenaceae*.

3.3 Archaea diversity

Hydrolysis, acidification and methanogenesis are several main steps for phenanthrene degradation under anaerobic methanogenic condition, and are often carried out by bacteria and methanogenic archaea (Yin et al., 2017). To clarify the archaeal groups responsible for methane production, an archaeal 16S rRNA gene sequencing was completed simultaneously. As shown in Fig. 3, the dominant archaea populations in the initial mixed samples were *Methanosarcina*, *vadinCA11* and *Methanobacterium*, constituting 34%, 28% and 21%, respectively. After enrichment phases, the relative abundances of *Methanosarcina* and *vadinCA11* have descended to less than 1% in the enrichment culture, while those of *Methanobacterium* and *Candidatus Nitrososphaera* have increased to more than 35% of the total archaeal sequences, representing 38% and 37%, respectively. The relative abundance of *Methanothermobacter* has grew to 21%, being the third most abundant in the enrichment culture. High relative abundance of *Methanosarcina* in the initial mixed samples might come from the anaerobic domestic sludge (Sekiguchi et al., 1998). The high relative abundance of *Candidatus Nitrososphaera* in the enrichment culture might result from much ammonium chloride provided in the nutrient medium. *Methanobacterium*, a hydrogenotrophic methanogen, often survives in the anaerobic sludge environments. Nearly all species in this genus could merely utilize hydrogen to produce methane. Thus, it is possible that hydrogen was an important metabolite during phenanthrene degradation for *Methanobacterium* growth. In other studies, Yuriy Kryachko also found that the microbial community in oil filed had a consistently high representative of hydrogenotrophs like *Methanobacterium* (Kryachko et al., 2012). Moreover, *Methanobacterium* was demonstrated to be predominant archaeal group in methanogenic phenanthrene-degrading culture developed from leachate-contaminated aquifer sediment (Zhang et al., 2012b) or from Baltimore Harbor sediments (Chang et al., 2008b). *Methanothermobacter* is also a hydrogenotrophic methanogen, often occurring in petroleum/gas reservoirs. For example, Gray reported that the archaeal members in

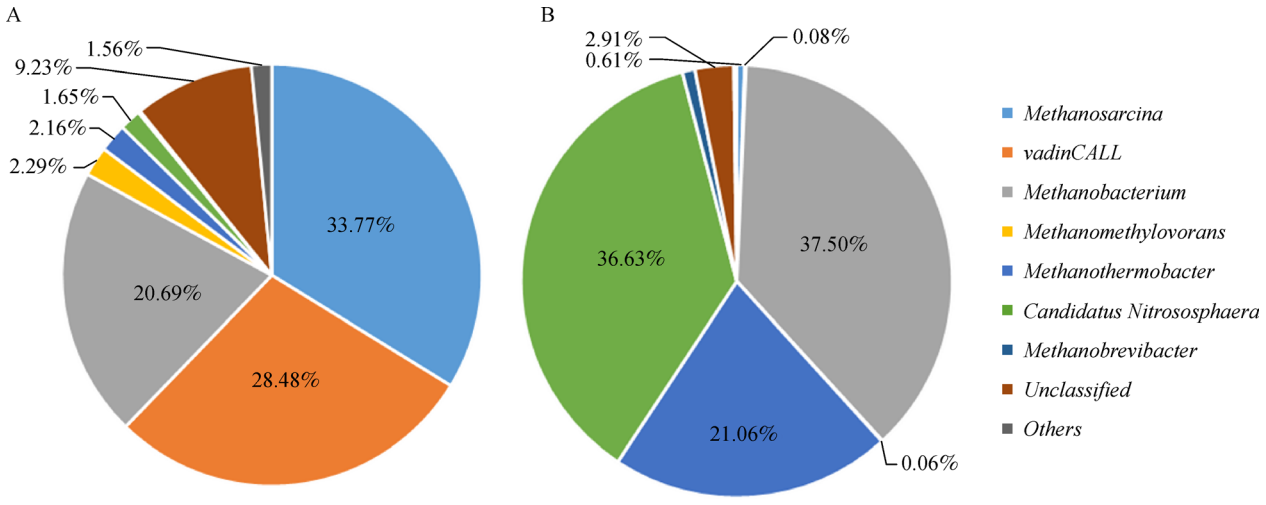

Fig. 3 Archaeal members at genus level of the initial sample and of the methanogenic enrichment culture; (A) Archaeal groups of initial mixed samples; (B) Archaeal groups of methanogenic enrichment culture.

an enrichment culture obtained from a large gas field in the North Sea were closely related to *Methanothermobacter* (Gray et al., 2009). Combined, the methanogenic archaea members responsible for methane production were two hydrogenotrophic methanogens: *Methanobacterium* and *Methanothermobacter*, which were similar to the ones in phenanthrene-degrading culture initiated from aquifer/harbor sediments.

3.4 Relationship between phenanthrene degradation and methane production

Here, the abilities of phenanthrene degradation and methane production in our enrichment culture were evaluated with batch experiments. As shown in Fig. 4, as phenanthrene degraded in the bottle, methane production gradually increased. The remaining amount of phenathrene at day 42 was 1.89 μmol, which meant that 3.55 μmol phenathrene was utilized; and simultaneously 0.5 μmol methane was detected in the bottle. According to Buswell's Eq. ($4C_{14}H_{10} + 46H_2O \rightarrow 33CH_4 + 23CO_2$), the theoretical carbon conversion rate to methane was 58.9% (33 carbon in methane were from 56 carbon in phenanthrene), however, the actual carbon conversion rate was only 1%. The low-efficiency of mixed culture to further degrade some metabolites into substrates methanogens could utilize, or the types of sustrates produced being unfavorable for the methanogens in our enrichment culture, might help to explain the low carbon conversion rate. Besides, in similar study, Berdugo-Clavijo showed that a naphthalene-degrading culture which had been enriched for over one year still produce small amount of methane (Berdugo-Clavijo et al., 2012).

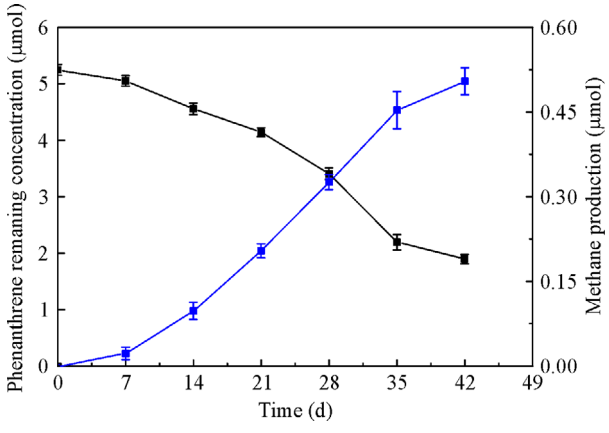

Fig. 4 Relationship of phenanthrene degradation and methane production.

3.5 Effects of different substances on phenanthrene degradation

Compared to aerobic degradation, PAHs degradation under anaerobic methanogenic conditon is a rather slow process. Table 1 demonstrates the effects of different substances on phenanthrene degradation by the enrichment culture.

Table 1 Substrate utilization tests

Substrate	degradation capability
Benzene	+
Naphthalene	-
2-methylnaphthalene	-
Phenanthrenecarboxylic acid	+
Phenanthrene	+
Anthracene	−
Fluoranthene	−
Pyrene	−

Note: + represents able; − indicates not able

Degradation data were well fitted to first-order kinetics (R^2: 0.94–0.97). In terms of hydrocarbons, hexane, hexadecane, benzene and phenanthrenecarboxylic acid noticeably enhanced phenanthrene degradation rate. Hexane and hexadecane are common components of hydrocarbons in oil-contaminated soil, which were reported to be the cometabolism substrates. Phenanthrenecarboxylic acid was reported to be the core metabolite during phenanthrene anaerobic methanogenic degradation, which could be one of metabolites in my culture.

It was interesting to note that the additon of sodium propionate and glucose increased degradation rates by 34.92% and 69.29%, while sodium acetate had no effects. The addition of glucose and acetate was reported to enhanced anaerobic PAH degradation in soil and sediment (Yuan and Chang, 2007; Chang et al., 2008a). In our enrichment culture, the dominant methanogen members (*Methanobacterium* and *Methanothermobacter*) are hydrogenotrophic. The degradation of propionate could produce hydrogen for methanogen growth. The neutral effects of acetate might be because few methanogens could directly utilize them. Similarly, fulvic acid and humic acid showed different effects on degradation rates. This could be explained by their dissimilar structure property. But the real mechanism needs to be further investigated.

Surfactants can increase PAHs solubility, but some of them also have toxic effects on microbes. Here, the addition of surfactants demonstrated contrasting effects compared to other studies. Both SDS and L-rhamnose showed positive impacts while Twain-80 had an opposite effect on phenanthrene degradation by this culture. In similar studies, SDS was demonstrated to change microbial community structure of a phenanthre-degrading culture under anaerobic methanogenic condition, thereby inhibiting phenanthrene degradation (Chang et al., 2008b). L-rhamnose was reported to show no positive effects on fluoranthene degradation by *Bacillus cereus* under anaerobic methanogenic condition (Fuchedzhieva et al., 2008). Thus, we could infer that SDS and L-rhamnose increased phenanthrene bioavailability and had no toxic effects on our culture, while Twain-80 might have changed microbial community structure in our culture.

As for other substances, the additon of BES inhibited phenanthrene degradation by 31.53% which was comparable to another study (Chang et al., 2006). Hydrogenotrophic methanogens was dominant (58.5%) in our enrichment culture, playing important role in methane production. Dolfing indicated that hydrogen threshold values below which complete oxidation of PAHs becomes exergonic under standard conditions are between log H_2 = –4.31 atm and log H_2 = –4.47 atm. Thus, hydrogen would accumulate when methanogenesis was inhibited, thereby making phenanthrene anaerobic degradation thermodynamically unfavorable. This result also implies a syntrophic cooperation necesaary for phenanthrene sustained degradation in our culture. The positive effects of magnetite and AQDS might be their role in promoting electron phase among microbes (Zhou et al., 2014; Li et al., 2015; Zhuang et al., 2015). However, the actual mechanism needs further study.

3.6 Substrate utilization

The substrate utilization diversity was tested with mono-aromatic and polycyclic aromatic hydrocarbons as the sole carbon source. As shown in Table 2, this culture was able to utilize benzene, phenanthrene and phenanthrenecarboxylic acid, but could not degrade other PAHs. This result was consistent with the positive effects of benzene and phenanthrenecarboxylic acid on phenanthrene degradation. It further indicated that phenanthrenecarboxylic acid could be a metabolite during phenanthrene degradation. The inability of our culture to utilize other PAHs showed that the microbes could not produce related enzymes to activate their first degradation reaction. As for PAHs aerobic degradation, microbes which can utilize higher molecular weight PAHs usually are able to degrade lower molecular weight PAHs. This is because most PAHs degradation will use the same set enzymes and genes to complete the initial activation reaction as well as the following degradation pathway. However, the initial activation reactions of different PAHs under anaerobic methangenic degradation might need different degraders and enzymes, and some studies have showed that different PAHs selected for different degraders (Chang et al., 2005; Zhang et al., 2012a;2012b). Furthermore, to date no

Table 2 Effects of different substances on phenanthrene degradation rate (K)

Treatment	K	K change
Inoculated control	0.031	
Hexane	0.053	67.87%
Hexadecane	0.062	96.60%
Benzene	0.052	65.39%
Phenanthrenecarboxylic acid	0.045	44.62%
Sodium propionate	0.042	34.92%
Sodium acetate	0.031	0.12%
Glucose	0.053	69.29%
Fulvic acid	0.031	−0.23%
Humic acid	0.039	24.18%
AQDS	0.045	42.57%
BES	0.021	−31.53%
Magnetite	0.039	23.22%
SDS	0.049	55.98%
L-rhamnose	0.048	54.34%
Twain-80	0.017	-47.42%

Note: AQDS represents anthraquinone-2,6-disulfonate, BES indicates sodium 2-bromoethanesulphonate, SDS represents sodium-dodecyl sulphate.

information about microbial community degrading fluoranthene and pyrene under anaerobic methangenic condition was available, which indicated that higher molecular weight PAHs were possibly difficult or even impossible to be utilized as carbon sources. The anaerobic degradation of them might be likely through co-metabolism pathway.

4 Conclusions

Phenanthrene degradation potential was noticeable in the mixed environment samples (oil-contaminated soil, coke wastewater and domestic sludge) which provide a range of diverse degraders compared to sediments. Bacterial members were mainly affiliated with *Comamonadaceae*, *Nocardiaceae* and *Thermodesulfobiaceae*, and methanogenic archaea groups were dominated by *Methanobacterium* and *Methanothermobacter*. Methane production was measurable but its amounts were far from stoichiometric. Batch experiments showed that different substances showed dissimilar effects on phenanthrene degradation, with some hydrocarbons and glucose having the most significant impacts. Lastly, substrate utilization tests indicated that this culture could not utilize other PAHs. All these analyses could bring some new insights for isolating PAH degraders and for removing PAHs from contaminated soil and sludge.

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