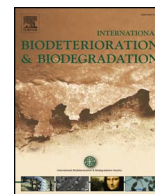




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Enhancing electron transfer by magnetite during phenanthrene anaerobic methanogenic degradation

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ABSTRACT

Recently, the potential of magnetite facilitating anaerobic methanogenic degradation of organic matters in natural or engineered microbial ecosystems has been reported. Here, we evaluated the effects of magnetite powder (< 5 μm) and nano Fe_3O_4 powder (50–100 nm) on phenanthrene methanogenic degradation by a mixed consortium enriched from petroleum contaminated soil. The results showed that complete phenanthrene degradation in our enrichment cultures required a syntrophic cooperation between degrading bacteria and methanogens. The addition of magnetite and nano Fe_3O_4 did not increase the relative abundance of methanogens, but improved phenanthrene degradation rates by 25.92% and 25.99%, and enhanced methane production rates by 21.5% and 20.1%, respectively. Further studies showed that the addition of magnetite and nano Fe_3O_4 improved electron transporting system (ETS) activities in the enrichment cultures, possibly through serving as electron conduits to facilitate DIET between syntrophic bacteria and methanogens.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a set of hydrocarbon compounds with two or more bonded aromatic rings (Menzie et al., 1992). Many of them are carcinogenic and/or mutagenic, and often persistently exist in the surroundings (Potin et al., 2004; Xu and Zhou, 2016). USEPA has listed 16 PAHs as priority control pollutants since 1976 (Bengtsson and Zerhouni, 2003; Keith and Telliard, 1979). Thus, it is important to remove them from the environment with the aim to keep people and wildlife from being threatened.

PAHs often exist in anoxic or anaerobic surroundings, such as aquifer sediment, harbor sediment and subsurface soil. Anaerobic degradation with nitrate, sulfate, Fe(III) or Mn(IV) as electron acceptors has been reported to be important processes for natural attenuation of PAHs in these environments (Anderson and Lovley, 1999; Chang et al., 2001a; Coates et al., 1996; Davidova et al., 2007; Mihelcic and Luthy, 1988; Rothermich et al., 2002). The initial activation of PAHs is essential for the further degradation. Carboxylation and activation by the addition of fumarate were two main first biochemical reactions for the degradation of alkanes (Mbadinga et al., 2011) and PAHs. Researchers reported that carboxylation was the initial biochemical step in the anaerobic metabolism of naphthalene and phenanthrene under sulfate-reducing condition (Davidova et al., 2007; Meckenstock et al., 2000; Zhang and Young, 1997), while the initial reaction of 2-methylnaphthalene degradation under sulfate-reducing condition involved

fumarate addition pathway (Meckenstock, 2000, 2002). Methanogenic condition can be formed through using carbon dioxide as electron acceptors because of the depletion of oxygen, nitrate, sulfate and metal ions (Zhang et al., 2012). PAH anaerobic degradation under methanogenic condition has also been reported (Chang et al., 2001b, 2005; Zhang et al., 2012), which indicated that methanogenic degradation might play a significant role in removing PAHs from our environment. Carolina detected 2-naphthoic acid and 6-methyl-2-naphthoic acid, the only metabolite in 2-methylnaphthalene-amended and 2, 6-dimethylnaphthalene-amended enrichment (Berdugo-Clavijo et al., 2012), respectively, which demonstrated that carboxylation was also an important biochemical pathway for methanogenic metabolism of methyl-substituted 2-ringed PAH. After initial activation, metabolites are gradually degraded by bacteria into acetate and hydrogen, then methanogens utilize these substrates to produce methane. For the process of methanogenic degradation, methanogenesis is considered to be the terminal electron accepting process (TEAP) (Chang et al., 2006). The observation that the degradation of phenanthrene and naphthalene by the enrichment cultures developed from harbor sediments declined when methanogenesis was terminated implied that a syntrophic cooperation in this microbial community was necessary for sustained PAH anaerobic oxidation (Chang et al., 2006). However, different habitats may select for different PAH degrading community (Wan et al., 2012), which might produce different types and quantity small molecule metabolites (like H_2/CO_2 , acetate only and H_2 plus acetate) converting

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into methane. Besides, Dolfig reported that phenanthrene methanogenic degradation would not necessarily be a syntrophic process which was assumed before (Dolfig et al., 2009). Thus, whether sustained PAH anaerobic methanogenic degradation requires a syntrophic process between the degrading bacteria enriched from other environments (like oil-contaminated soil) and methanogens needs further study.

Interspecies electron transfer (IET) was the key process for syntrophic methane production (Stams and Plugge, 2009), and it can be completed through the diffusion of hydrogen or formate, which was previously assumed the sole mechanism for IET. Recently, direct interspecies electron transfer (DIET) (Li et al., 2015) between syntrophic partners has been reported to be of great significance. Rotaru demonstrated that in the oxidation of ethanol to methane, *Geobacter metallireducens* shared electrons with *Methanosarcina barkeri* (Rotaru et al., 2014b) or *Methanosaeta harundinacea* (Rotaru et al., 2014a) through conductive pili and c-type cytochrome. In addition, in microbial aggregates derived from an upflow anaerobic sludge blanket reactor, a pili-dependent DIET occurred indicated by some powerful evidence (Morita et al., 2011). More interestingly, carbon-based and iron-based conductive materials have been reported to be a good substitution for pili or outer membrane c-type cytochromes (OmcS), playing great roles as electron conduits in the long-distance extracellular electron transport. For example, Rotaru demonstrated that the pilin-deficient *Geobacter metallireducens* was still able to transfer electrons to *Methanosarcina barkeri* during the oxidation of ethanol to methane due to the addition of granular activated carbon (Rotaru et al., 2014b). In spite of the absence of pilin-related c-type cytochrome (OmcS), DIET in *Geobacter* co-cultures could be mediated by magnetite, thereby completing electron transfer during the process of substrate conversion (Liu et al., 2015). In addition to playing significant roles in co-culture systems, conductive materials have been reported to have positive effects on the conversion of organic matters (acetate, ethanol, butyrate and benzoate) to methane in paddy soils (Kato et al., 2012a,b; Li et al., 2015; Zhou et al., 2014; Zhuang et al., 2015), and on the conversion of other organic matters (propionate, artificial complex waste, and synthetic wastewater) to methane in anaerobic sludge digester (Cruz Viggli et al., 2014; Yin et al., 2016; Zhao et al., 2016). The back mechanism of conductive materials improving the conversion of organic matters into methane has been reported to be the improved electron transfer between substrate-oxidizing bacteria and methanogens facilitated by conductive materials.

To my knowledge, the organic matters previously provided were either favorable substrates (e.g. acetate, ethanol, propionate and butyrate) or contained some favorable substrates (like sodium acetate in synthetic wastewater) for syntrophic community. PAHs are a group of persistent organic pollutants, unfavorable substrates for syntrophic partners. And anaerobic degradation of PAHs also requires some specific degrading bacteria, like *Citrobacter* and *Pseudomonas* reported by Zhang (Zhang et al., 2012). However, the potential of conductive materials for improving PAH methanogenic degradation and the possibility of DIET occurring in such process have not yet been reported.

In the present study, a phenanthrene-degrading microbial consortium was developed from petroleum-contaminated soil under methanogenic condition, micrometer-size ($< 5 \mu\text{m}$) Fe_3O_4 powder (magnetite) and nanometer-size (50–100 nm) Fe_3O_4 powder (nano Fe_3O_4) were selected as conductive materials due to their abundance in natural systems. The potential of magnetite for facilitating phenanthrene methanogenic degradation was firstly explored, which is beneficial to remove PAH from the environment. Then we investigated the necessity of syntrophic cooperation in this microbial community for sustained phenanthrene degradation and the possibility of DIET in the process of this consortium converting phenanthrene into methane. We finally explored the effects of magnetite and nano Fe_3O_4 on methanogen abundance during incubation time. These analyses can bring us better understanding of the important role of conductive iron oxides in PAH natural attenuation occurring in contaminated sites.

2. Methods and materials

2.1. A phenanthrene-degrading consortium

A methanogenic phenanthrene-degrading consortium was enriched from petroleum contaminated soil sampled in Shengli Oil Field, China. The initial enrichment was carried out in a 0.5 l beaker flask placed on a six-header agitator, starting with soil (30 g wet weight/L) as the microbial inoculum, 20–30 mg l^{-1} phenanthrene as the sole substrate, and nutrient medium containing sodium bicarbonate (20 mM) made up the remaining volume. The reactor was run at 32 °C and gently stirred with a magnetic stir. The nutrient medium (per liter of water) followed a previous study (Chang et al., 2002) with some modifications: 1.2 g NH_4Cl , 0.27 g K_2HPO_4 , 0.1 g CaCl_2 , 0.35 g KH_2PO_4 , 0.1 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.02 g $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 0.0001 g resazurin, 0.02 g citric acid-titanium chelate, 1 mL trace element solution and 0.2 mL vitamin solution. The trace element solution and the vitamin solution referred to another study (Zhang and Young, 1997). To remove oxygen, the flask was bubbled with N_2 for 20 min, and was supplemented with 10 mg sodium sulphide. Medium pH was adjusted to 7.2–7.4 with hydrochloric acid and sodium hydroxide. Finally, the rubber stopper with predrilled holes connecting three tubes (one for regular sampling, one for collecting gas and one for controlling temperature) was used to seal the flask. When half or more phenanthrene was consumed, the consortium was transferred into fresh nutrient solution and phenanthrene was supplemented to 20–30 mg l^{-1} . A methanogenic degradation consortium was obtained after 9–10 transfers.

2.2. Batch experiments

200 mL anaerobic serum bottles were used to conduct the batch experiments, each containing 8 mL anaerobic phenanthrene-degrading consortium (OD = 1.5) and 32 mL nutrient medium described previously, spiked with 25 mg l^{-1} phenanthrene. Magnetite powder and nano Fe_3O_4 (50–100 nm) powder purchased from Beijing Dingguo Changsheng Biotechnology Company were given the ultimate concentration of 10 mM as Fe atoms, respectively. At the same time, bottles without magnetite and nano Fe_3O_4 , and bottles containing no phenanthrene-degrading consortium were set as control treatment and abiotic control, respectively. All bottles were bubbled with N_2 for 5 min and was spiked with 1 mg sodium sulphide that helps to consume the remaining oxygen in the medium. Then pH was adjusted to 7.2–7.4. Finally, all anaerobic bottles were capped with butyl rubber stoppers (Zheng et al., 2015) and incubated on a horizontal shaker (32 °C and 165 rpm). At regular time, triplicate bottles were taken out to measure the concentrations of Fe(II) and phenanthrene, and methane production.

2.3. Analytical methods

Methane in the bottles was measured with gas chromatograph (GC-2014). At regular time, we used a sterile syringe to inject 1 mL of incubation headspace into GC equipped with a thermal conductance detector. The measure conditions were as follow (carrier gas: high purity nitrogen; chromatographic column and detector temperature: 100 °C; oven: 330 °C). Carbon dioxide was determined with the same method of methane measurement. Phenanthrene was extracted with equal-volume methylene chloride and measured with high performance liquid chromatograph (HPLC) described previously (Fang et al., 2016). The acid-soluble Fe(II) concentrations were analyzed by the ferrozine technique described by Lovley (Lovley and Phillips., 1986). Dissolved organic carbon (DOC) in the supernatant was determined with measuring carbon dioxide converted through combustion at 680 °C as well as catalytic oxidation (TOC-VCPH), the actual DOC in the supernatant equals the measured DOC minus dissolved phenanthrene (about 1 mg l^{-1} : 3.14 $\mu\text{mol carbon}$). To examine the chemical stability, both

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