Co-variations of bacterial composition and catabolic genes related to PAH degradation in a produced water treatment system consisting of successive anoxic and aerobic units

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Abstract

This paper reports on the investigation of concentration levels of PAHs, community structure, as well as the abundance of PAH-related catabolic genes including upper-pathway dioxygenase genes (*nahAc* and *phnAc*) and down-pathway catechol dioxygenase genes (*C12O* and *C23O*) in a successive anoxic and aerobic treatment of produced water from the Jidong Oilfield, China. 93% of total PAHs were removed, almost equally contributed by the anoxic and aerobic units. However, PAHs of more than 3 benzene rings remained almost unchanged. The signals for *phnAc* and *C12O* were undetectable in this biological system, whereas the existence of *nahAc* and *C23O* was confirmed in the system and the copies of the two genes in the aerobic tank were 2 or 3 orders higher than those in the influent water sample. The different behavior of *C23O* demonstrated that mineralization of PAHs might mainly occur in the aerobic unit. The existence of *nahAc* and *C23O* genes in the influent and the high similarity of genotype between the influent and the two sludge samples suggested that bacteria existing in the influent contributed to PAH removal and bacteria harboring PAH catabolic genes were enriched in the sludge.

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1. Introduction

The metabolism of PAHs in pure culture and transformation of PAHs in mixed microbial communities have been studied for many years (Cerniglia, 1992). A wide varieties of microorganism resources have been

found to be available for PAH degradation (Grishchenkov et al., 2000; Pan et al., 2004). Biochemical pathways for many lower-molecular-weight PAHs (LMW PAHs) and their biodegradation mechanisms have been clarified. Most LMW PAHs could be decomposed through direct biological degradation (Moody et al., 2001). The high-molecular-weight PAHs (HMW PAHs), on the other hand, used to be considered as being resistant to biodegradation. Recent reports indicated that many

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microorganisms could degrade HMW PAHs of 4 to 5 benzene rings through co-metabolism or other mechanisms (Moody et al., 2004; Rentz et al., 2005). With the evolution of microorganisms and progress of wide range studies, more and more PAHs have been found to be biodegradable (Hamme et al., 2003). Genetic analyses of PAH catabolic pathways in several PAH-degrading bacteria revealed the presence of a group of genes for degradation of aromatic compounds. The genes encoding the dioxygenase and catechol cleavage dioxygenase were found to be responsible for the rate-limiting step during LMW PAH degradation (Hamme et al., 2003; Onruthai et al., 2004), and the abundance of the related catabolic genes in a biological system could reflect the degradation potential of the compounds (Tuomi et al., 2004). While great progress has been achieved in studies of PAH degradation, almost all of the previous PAH-related results were obtained in simulated biological systems or pure cultures (Cullen et al., 1994; Tian et al., 2002), making the environmental meaning of the results to be limited.

The oilfield produced water, which accompanies with crude oil exploration and production, has long been known as one of the largest waterborne PAH pollution sources. Compared with other industrial wastewaters, the oilfield produced water is characterized with the high temperature, high salt concentration and containing many other hydrophobic substances (Telleza et al., 2002; Hamme et al., 2003). In most cases, the produced water had to be properly treated to meet the discharge standards on chemical oxygen demand (COD) and oil content before being discharged into the receiving water bodies. However, information on the removal of PAHs from the pollutant sources like produced water, especially in the existing biological treatment systems, is minimal even though it is very important for establishing wastewater treatment strategies or standards to control waterborne PAH pollution.

In this study, the performance of an existing produced water treatment system consisting of successive anoxic and aerobic biological units was evaluated by choosing PAHs as the target pollutants. The abundance of related functional genes like upper-pathway dioxygenase genes (*nahAc* and *phnAc*) and down-pathway catechol dioxygenase genes (*C12O* and *C23O*) from the influent and the sludge samples of the two reactors was monitored using most probable number polymerase chain reaction (MPN-PCR) to help understanding the biodegradation mechanisms of PAHs, and the bacterial community structure was analyzed using the denaturing gradient gel electrophoresis (DGGE) of the PCR amplified 16S rDNA V3 region. The change of PAH level was correlated with the

changes of the catabolic genes and biodiversity in the existing biological treatment system.

2. Materials and methods

2.1. Sample collection

The biological treatment system with a capacity of 5000 tons of produced water per day is located in Jidong Oilfield, Hebei Province, China. It was constructed and put into use in 2001. The system consists of two identical series-wound tanks (30 m × 18 m × 5.5 m) filled with fiber type biocarrier. Only the second tank is aerated, and the hydraulic retention time (HRT) of each reactor is about 12 h. The redox potential (ORP) in the water depth of 40 cm from the surface of the first reactor was −130 mV, indicating an anoxic state. The volumetric COD load of the system is 0.72–0.75 g l^{−1} d^{−1}. Sloughed biofilm is discharged without returning to the system. For PAH analysis, 4 l water samples were collected from the influent and the effluents of the anoxic and aerobic units, respectively, in clean brown glass bottles in March 2005. Simultaneously, the influent and sloughed biofilm samples from the bottoms of the two reactors were collected in sterile bottles for DNA extraction. All the samples were processed in the laboratory within 12 h after sampling.

2.2. Quantification of PAHs and water quality analysis

Water samples (4 l) were taken over a period of 24 h according to the protocol of US Environmental Protection Agency (USEPA) and were processed immediately upon arrival in the laboratory (Chen et al., 2005). The concentrations of PAHs in the extracts were determined by an Agilent 6890GC equipped with a 5973 mass selective detector using an HP-5 silica fused capillary column (60 m × 0.25 mm × 0.25 μm film thickness) under the selected ion monitoring mode. The recoveries for surrogate standards fell within a fairly narrow range and, for individual PAHs, were between 73.2 ± 5.6% and 90.3 ± 6.2%. The conventional water quality parameters of the influent and the effluents of the two reactors were measured according to the standard methods (Andrew et al., 1998).

2.3. PCR primers and conditions

PCR primers, as shown in Table 1, were chosen from related published references and the conditions were optimized before experiments. The optimized PCR mixtures contained 10 mM of Tris-HCl, 50 mM of

Table 1
Primers used in this study

Primer	Target	Sequence (5'–3')	Expected product size (bp)	Reference
<i>EUB</i> -F	16s rDNA	GCACAAGCGGTGGAGCATGTGG	455	Iwamoto et al. (2000)
<i>EUB</i> -R		GCCCCGGAACGTATTCACCG		
<i>EUB</i> 341-F	16s rDNA	CCTACGGGAGGCAGCAG	230	Muyzer et al. (1993)
<i>EUB</i> 518-R		ATTACCGCGGCTGCTGG		
<i>phnAc</i> -F	phnAc	TTCGAGCTGGAATGTGAGC	993	Laurie and Jones (2000)
<i>phnAc</i> -R		AATAACCGGCGATTCCAAAC		
<i>nahAc</i> -F	nahAc	TGGCGATGAAGAACTTTTC	992	Laurie and Jones (2000)
<i>nahAc</i> -R		AACGTACGCTGAACCGAGTC		
<i>C12O</i> -F	C12O	GCCAACGTCGACGTCTGGCA	282	Sei et al. (1999)
<i>C12O</i> -R		CGCCTTCAAAGTTGATCTGCGTGGT		
<i>C23O</i> -F	C23O	AAGAGGCATGGGGGCGCACCGGTTTCGATCA		
<i>C23O</i> -R		CCAGCAAACACCTCGTTGCGGTTGCC	377	Sei et al. (1999)

GC-clamp, CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G was attached to *EUB*341-F for DGGE.

KCl, 2.5 mM of MgCl₂, 0.2 mM of each dNTPs, 0.2 mM of the primers, 1.25 U Taq-polymerase (Takara, China) and 2 µl of the extracted DNA template. All PCR were made in triplet.

2.4. Total DNA extraction from samples

The two sloughed biofilm samples (0.05 g) and the influent water sample (1.0 g) were centrifuged at 8000 rpm for 5 min. Each solid residue was washed twice by phosphorous buffer solution and re-suspended in 400 µl of phosphorous salt buffer. Followed by adding 400 µl of breaking buffer (1% SDS, 100 mM NaCl, 10 mM Tris with pH at 8.0, 1 mM EDTA with pH at 8.0) and 600 µl of phenol-chloroform (v/v=25:24). The cells were then homogenized by vortexing and incubated at 60 °C for 10 min. After centrifugation, the supernatant was extracted once with 1 volume of phenol-chloroform-isoamyl alcohol (v/v/v=25:24:1, pH=8.0) and once with 1 volume of chloroform-isoamyl alcohol (v/v=24:1, pH=8.0). Nucleic acids were precipitated by adding 0.1 volume of sodium acetate (pH=5.2) and 0.6 volume of isopropanol to the supernatant. The mixture was incubated in ice for 30 min and centrifuged (15,000 g for 20 min at 4 °C) to recover the precipitated nucleic acids. The pellets were washed with 70% ice-cold ethanol and centrifuged (15,000 g for 20 min at 4 °C). The pellets were dissolved in 100 µl of TE buffer after air drying. The DNA was purified using a UNIQ-10 column purification kit (Sangon, China) according to the manufacturer's instructions.

2.5. DNA enumeration by MPN-PCR

The purified DNA was serially diluted by 10-fold, and three samples of each dilution step were subjected to

PCR. The programs of touchdown PCR were optimized as follows: first denaturation at 94 °C for 4 min., then 40 cycles with denaturation at 94 °C for 40 s. The initial annealing temperature was set at 65 °C and was then decreased by 0.5 °C in each cycle until it reached 60 °C. Thirty additional cycles were applied for *C12O*, *C23O* and *EUB* amplification. For *nahAc* and *phnAc*, the annealing temperature was from 57 °C to 52 °C with a decrease of 0.5 °C in each cycle (10 cycles). The annealing temperature for the additional 30 cycles was 52 °C with elongation at 72 °C for 40 s and a final elongation at 72 °C for 10 min. The PCR products (10 µl) were analyzed by electrophoreses on a 1.2% agarose gel. The gel was stained with 0.5 µg ml⁻¹ of ethidium bromide solution, and photographed under UV light after being rinsed with distilled water.

2.6. PCR-DGGE and biodiversity analysis

The V3 region of the 16S rRNA gene was amplified by PCR using the forward primers *EUB*341F with a GC clamp and the reverse primer *EUB*518R. The touchdown PCR (Thermal Cycler, Bio-Rad, USA) was performed as follows. Initial denaturation was at 95 °C for 5 min. Thirty five cycles with denaturation were carried out at 94 °C for 40 s. The annealing temperature was initially set at 62.5 °C for 40 s and was then decreased by 0.5 °C in each cycle until it reached 55 °C. Twenty additional cycles were carried out at 55 °C. Elongation was carried out at 72 °C for 40 s, and a final elongation was carried out at 72 °C for 10 min. Five ml of the amplified mixture was analyzed in a 1.5% 0.5×TBE agarose gel electrophoresis. The gel was stained with 0.5 mg ml⁻¹ ethidium bromide, rinsed with distilled water and photographed. For PCR product sequence specific separation, DGGE was performed

Table 2
Quality of the influent and effluent of produced water

Parameter	Value	
	Influent	Effluent
pH	7.3±0.2	7.3±0.2
Temperature, °C	60±2	50±2
Total dissolved solids, mg l ⁻¹	5560±35	5400±35
Total petroleum hydrocarbons, mg l ⁻¹	23±3.5	2±0.3
Oil and grease, mg l ⁻¹	28±5.5	2±0.3
COD, mg l ⁻¹	470±25	65±5
BTEX mg l ⁻¹	650±25	20±2
NH ₄ ⁺ -N mg l ⁻¹	8.10±0.6	8.0±0.4
NO ₃ ⁻ -N mg l ⁻¹	0.80±0.19	0.40±0.1

with a Bio-Rad Dcode System (Bio-Rad, USA). The PCR product was loaded on 0.8 mm thick polyacrylamide gel (10% w/v acrylamide: bisacrylamide 37.5:1) using a denaturing gradient from 30% to 50% of urea and formamide (100% corresponds to 7 M urea and 40% w/v formamide) being increased in the direction of the electrophoresis run. The electrophoresis was performed under a constant voltage of 150 V for 5 h and a constant temperature of 60 °C. After the run, the gels were stained for 20 min in 1×TAE containing 0.5 mg ml⁻¹ ethidium bromide and rinsed with distilled water, then photographed. Diversity analysis included the number of bands (I), the Shannon–Weaver index (H). Community similarities were quantified using the Dice index (Cs) (Sun et al., 2004; Bento et al., 2005).

3. Results and discussion

3.1. Performance of the produced water treatment system

The quality of the oilfield produced water before and after biological treatment is shown in Table 2. It is clear

that the biological system was quite efficient in removing the conventional pollutants such as COD, SS, BTEX, and oil contents. The COD in the final effluent was 60–70 mg l⁻¹. The removal ratios of both oil and petroleum hydrocarbons were about 90%, and the removal ratio of BTEX was more than 90%.

In this study, all the 16 PAHs included in the USEPA's priority pollutant list and parts of alkyl-substituted PAHs were analyzed as the target substances. Changes of the total PAHs, LMW PAHs and HMW PAHs are shown in Fig. 1(A) and (B), respectively. About 93% of the total PAHs in the influent were removed through the biological treatment. The contributions by the anoxic and aerobic treatment were 45% and 48%, respectively, indicating that PAHs could be removed under anoxic conditions. Recently, several research groups reported that many microorganisms had the ability to degrade HMW PAHs through co-metabolism with laboratory prepared samples (Bouchez et al., 1995; Moody et al., 2004; Pan et al., 2004; Rentz et al., 2005). However, as shown in Fig. 1(B), the HMW PAHs had no obvious change during the biological wastewater treatment process. Only naphthalene, phenanthrene, and the alkyl-substituted two or three-ring PAHs were removed efficiently (Fig. 1A). It is clear that the conditions of the biological treatment system did not favor biodegradation of the HMW PAHs. One possible reason might be that an HRT of 24 h was not sufficient for the biodegradation of the HMW PAHs. In relation to the levels of LMW PAHs, changes of catabolic gene copies and biodiversity of the influent and sludge samples were further analyzed by the molecular techniques.

3.2. Enumeration of catabolic genes for PAH degradation

The catabolic genes can be used to trace the specific activities of bacterial populations. We targeted several

Fig. 1. Levels of total PAHs, LMW PAHs (A): (▨) total PAHs, (▩) LMW PAHs, and HMW PAHs (B) during the treatment.

Fig. 2. Catabolic genes enumeration by MPN-PCR (A): (▨) *EUB*, (□) *nahAc*, (▤) *C23O* and ratios of copy numbers of catabolic genes to those of *EUB* in different samples (B): (♦) *nahAc*/*EUB*; (■) *C23O*/*EUB*.

key enzyme coding genes (dioxygenase) responsible for the rate-limiting steps during the mineralization of PAHs. No signals for *phnAc* and *C12O* were detected in all of the three samples. The existence of *nahAc* and *C23O* were confirmed, and the results are shown in Fig. 2(A). It is interesting that both of the two genes existed even in the influent water although the copies of genes were relatively low. The copies of the two genes in the aerobic tank were 2 or 3 orders higher than those in the influent water sample. The ratios of the copy numbers of the catabolic genes to that of the *EUB* genes, which could represent the enrichment of functional bacteria, are shown in Fig. 2(B). It is interesting that the *nahAc* gene increased in the anoxic tank, which could

be accounted for the PAH removal in it, as shown in Fig. 1(A). The *C23O* gene, however, remained almost unchanged, suggesting that mineralization of PAHs did not occur in the anoxic tank. On the other hand, the two genes increased significantly in the aerobic tank, which indicates that mineralization of the LMW PAHs was possibly realized in the aerobic degradation.

Laurie and Jones (2000) reported that the *phn* genotype would have greater ecological significance than the *nah*-like genotype in a soil environment. But our result suggested that the *nah*-like genotype was more important for removing the LMW PAHs from produced water. This result was in accordance with our lab scale experimental systems. Results from other research groups also testified to a ubiquitous distribution of the *nahAc* genotype in bilge waste, petroleum waste sludge, and soil contaminated with creosote and petroleum products (Mavrodi et al., 2003; Olivera et al., 2003; Katsivela et al., 2005). It is possible that oil-polluted environments containing a relatively high PAH concentration favored the *nahAc* genotype, while

Fig. 3. DGGE profile (A) and pattern (B) of 16S r DNA V3 region. (1) aerobic sludge, (2) anoxic sludge, and (3) influent.

Table 3
Diversity indices between communities within three samples

Sample	Diversity indices			
	<i>I</i>	<i>H</i>	<i>S</i>	<i>Cs</i> (%)
1#	12	2.44	0.36	70
2#	13	2.50	0.34	75
3#	13	2.55	0.27	100

(*I*) number of bands; (*H*): $\sum P_i \ln[P_i]$, where P_i is the intensity of a given band divided by the total intensity of bands detected; (*S*): Ratio of sum of three most dominant bands (intensity) to the total bands intensity; (*Cs*): $Cs = 2j / (a + b)$, where j is the number of bands common to influent samples, a and b are the number of bands in different samples; 1# from aerobic sludge, 2# from anoxic sludge, and 3# from influent.

the less polluted environments with a low PAH concentration favored the *phn*-like genotype.

During the mineralization of PAHs, *C12O* and *C23O* were two key genes responsible for aromatics cleavage. The results indicated that the *C23O* gene was ubiquitous during the treatment process. However, no signal of *C12O* was detected. Sei et al. (2004) proved that *C12O* was primarily responsible for phenol and benzoate degradation at low concentrations in seawater microcosms, while bacteria containing *C23O* were enriched under high-load condition for relatively long periods. Thus, it was reasonable that *C23O* instead of *C12O* was the dominant gene for aromatics cleavage in this existing treatment system with higher BTEX contents.

3.3. Biodiversity analyzed by DGGE

PCR-DGGE with the bacteria-specific primers of *EUB341F*-GC and *EUB518R* permitted us to detect the predominant bacteria species. The obvious difference of DGGE banding patterns of the three samples is exhibited in Fig. 3. The related indices of diversity were calculated from the results of PCR-DGGE, and are shown in Table 3. The differences of H' and band number were not so significant among the three samples. Slight decreases of H' during the treatment were observed. From each sample, the sum of the three most dominant bands (intensity) divided by the total intensity of bands detected was calculated (Sun et al., 2004), and the resulting values for the influent, anoxic sludge and aerobic sludge were 0.27, 0.34 and 0.36, respectively. The increased values of the sludge samples indicated that the dominating bacteria were enriched in the treatment system, and pollutant degradation might be mainly contributed by several dominant species.

The similarity of DGGE bands between the influent sample and the two sludge samples were 75% and 70%, respectively, which indicated that bacteria from the influent were viable in the biological system and played an important role in pollutant degradation. This was also supported by the existence of catabolic genes in the influent sample.

4. Conclusions

A biological treatment system consisting of successive anoxic and aerobic units can efficiently remove the LMW PAHs from the produced water. The contribution of the aerobic process to PAH removal was similar with that of the anoxic process. But the system was not able to

remove PAHs with 4 benzene rings or more. Biodegradation of PAHs from the produced water was further confirmed by the existence of high copy numbers of *nahAc* and *C23O* in the biological treatment system.

Different dominant communities were enriched in different treatment units. The high similarity of genotype between the influent and sludge bacteria from two reactors, and also the existence of the catabolic genes in the influent indicates that many of the bacteria from the influent contribute to the degradation of pollutants. The sources and composition of bacteria in the influent produced water is of interest, and will be investigated in future studies.

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