Environ Earth Sci (2016) 75:119
DOI 10.1007/s12665-015-4787-7

ORIGINAL ARTICLE

^{14}C Isotopes and microbial community structures as evidence for biodegradation in a petroleum hydrocarbon-contaminated aquifer

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Received: 26 December 2014 / Accepted: 8 July 2015 / Published online: 6 January 2016
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Abstract Petroleum hydrocarbon contamination of groundwater and soil constitutes a serious threat to human health and the natural environment. In situ biodegradation is a low-cost, low-risk and effective technique to remove petroleum hydrocarbon contaminants. The concentrations of total petroleum hydrocarbons (TPH), dissolved inorganic carbon (DIC) and $^{14}C_{HCO_3}$ were analyzed, and polymerase chain reaction–denaturing gradient gel electrophoresis was used to investigate bacterial community structures. Downgradient from the contaminated source along the groundwater flow, the concentrations of TPH and DIC decreased and the ^{14}C content increased from 44.3 to 64.3 pmc, which confirmed petroleum hydrocarbon biodegradation in the contaminated aquifers. The sequence analysis of the DGGE bands revealed that some species of known bacterial biodegrading strains, such as *Dechloromonas aromatica* RCB, *Pseudomonas putida* and *Pseudomonas protegens*, were the dominant bacterial groups in the field. Hydrogeochemical and stable isotope measurements, combined with analysis of bacterial community structures, could be useful tools to prove the occurrence of biodegradation processes in contaminated aquifers.

Keywords Groundwater · Petroleum hydrocarbon contamination · ^{14}C · Microbial community structures

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Introduction

Petroleum hydrocarbon (PHC) spills are some of the most widespread causes of soil and groundwater contamination (Conrad et al. 1997). Biodegradation is the only process included under the definition of natural attenuation that leads to a reduction in the total mass of PHC (Bolliger et al. 1999). Detailed investigation and demonstration are needed to ensure that the contamination load is reduced. Evidence for the microbial degradation process is commonly acquired by monitoring the decrease in contaminant concentrations (Douglas et al. 1992), monitoring electron acceptors, measuring increases in the degradation products (Borden et al. 1995; Anderson and Lovley 1997; Lesage et al. 1997; Kao and Wang 2001) and measuring biomass concentrations (Madsen et al. 1991; Fleming et al. 1993). However, effects that are similar to those expected from contaminant biodegradation (e.g., the biodegradation of organic matter or the dissolution of carbonate minerals) can occur for other reasons, often giving ambiguous results.

The microbial metabolism of compounds derived from PHC tends to produce CO_2 with relatively low ^{13}C values because hydrocarbon compounds are generally depleted in ^{13}C relative to most other sources of C (Schoell 1984). Stable carbon isotope analysis has been applied to identify biodegradation byproducts and to provide further verification of petroleum biodegradation in groundwater and vadose zone gases (Aggarwal and Hinchee 1991; National Research Council 1993; Bolliger et al. 1999; Hunkeler et al. 1999; Fang et al. 2000; Topinkova et al. 2007). Definitive confirmation of biodegradation might be hindered when contaminant and natural organic carbon stable isotope ratios overlap. For example, in a system that has become anaerobic because of contaminant inputs, methane production yields ^{13}C-depleted CH_4 and

^{13}C-enriched CO_2 (Lollar et al. 2001), so the δ^{13}C values for CO_2 and/or DIC produced from degradation of PHC in areas of significant methanogenic activity can be higher rather than lower than expected from hydrocarbon degradation alone (Baedecker et al. 1993; Revesz et al. 1995; Landmeyer et al. 1996). There can also be significant overlaps between the δ^{13}C values of PHC contaminants and indigenous plants. In such areas, natural abundance radiocarbon analysis is an alternative approach for tracing carbon assimilation and respiration through aerobic petroleum mineralization (Aelion et al. 1997).

The ^{14}C content can be used to distinguish between carbon sources of different ages (Bauer et al. 1990, 1992, 1995) because it has a half-life of approximately 5680 years. PHC of geological origins are, therefore, radiocarbon-free (0 pmc) and provide a definitive end-member when analyzed against photosynthesis-based carbon-containing chemicals, which contain modern CO_2 from the atmosphere (which is approximately 110 pmc; Conrad et al. 1997). Given these two extreme end-members, one can readily assess the relative contributions of petroleum and plant biomass degradation to the total respired CO_2 pool (Suchomel et al. 1990; Bhupathiraju et al. 2002; Conrad and Depaolo 2004; Bugna et al. 2005). In situ ^{14}C and ^{13}C measurements have been used in several studies under methanogenic environmental conditions (Coffin et al. 2008).

In the process of microbial remediation of environmental contamination, the presence of microorganisms with the ability to degrade the target contaminant(s) is undoubtedly the key element. There are several methods available to categorize the microbial community, including restriction fragment length polymorphisms, single-stranded conformation polymorphism and denaturing gradient gel electrophoresis (DGGE). DGGE is a rapid and efficient separation technique of DNA sequences of the same length (amplified by PCR) and has been utilized in diverse subject areas such as clinical and environmental microbiology (Mouser et al. 2005; Kaoa et al. 2010). Rarely has DGGE been combined with hydrogeochemical and radioactive isotope analyses to identify biodegradation processes.

The shallow aquifer of the study site was contaminated by PHC because of an aquiclude penetration of oil from oil wells. In our previous study, the existence of biodegradation processes was confirmed by analysis of hydrogeochemical and stable carbon indicators (Su et al. 2013). It could not be determined whether the more positive δ^{13}C value of DIC in the groundwater located near the contaminated source was influenced by the biodegradation process. Here, groundwater samples were collected and analyzed for ^{14}C isotope and microbial community structures, with the objectives of (1) demonstrating the effectiveness of combined microbial community structures and ^{14}C measurements in identifying the biodegradation of TPH and (2) better understanding of biogeochemical conditions of TPH.

Materials and methods

Site description

The shallow aquifer of the study site was contaminated by PHC because of aquiclude penetration of oil from oil wells. The quaternary unconsolidated rock pore water aquifer is the main water supplying strata. Figure 1 shows that the lithology and thickness of the shallow formations are relatively stable. From top to bottom, the aquifer lithology is successively fine sand, silt and gravel, and the total thickness is about 20 m. There is a 2-m impermeable clay layer above the aquifer, and the lower part of the silt clay layer forms the bottom of the aquifer. Because of the relatively closed structure of the shallow confined aquifer, precipitation and air cannot readily infiltrate the clay layer, and the downward migration of contaminant on the land surface is restricted. The main source of aquifer recharge is the lateral runoff from hillock groundwater, and the aquifer is discharged by lateral runoff and artificial extraction. Groundwater level measurements indicate that the depth of the groundwater table is about 3.5 m and the direction of groundwater flow is from southeast to northwest with a hydraulic gradient of 0.5 ‰.

Data from all monitored wells showed that the groundwater was contaminated by PHC. The groundwater-contaminated plume area is about 3600 m^2. The extension of the total petroleum hydrocarbon (TPH) plume reaches a steady-state distribution, and the spreading direction of the plume is controlled by the groundwater flow, which is from southeast to northwest. The degree of groundwater pollution is the most severe near the abandoned oil well and gradually decreases along the direction of groundwater flow (as shown in Fig. 2). The content of groundwater TPH is less than 0.05 mg/L in wells Z10, Z9 and Z11 (Su et al. 2013).

Sampling and analysis

Sampling

There were 19 monitoring wells at the contaminated site. Groundwater and soil samples were collected in November 2011, and the main parameters measured were TPH, organic and inorganic chemical compositions, pH, oxidation–reduction potential (E_h) and dissolved oxygen (DO). Groundwater samples for the analysis of microbial community structures were also collected from all wells. Seven

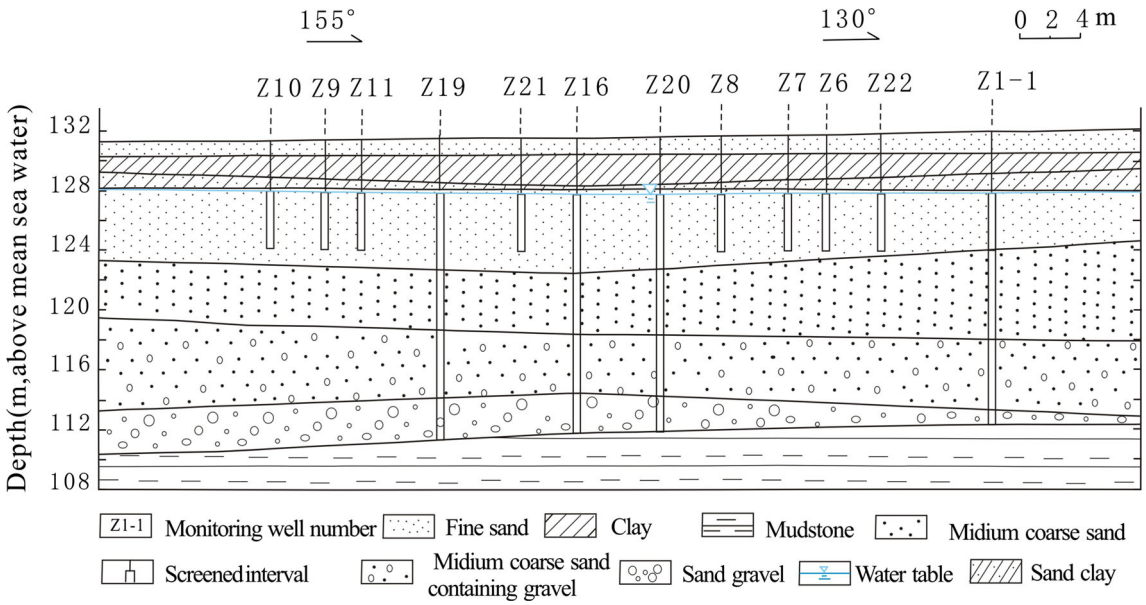

Fig. 1 Hydrogeological cross-sectional sketch of I–I' (Su et al. 2013)

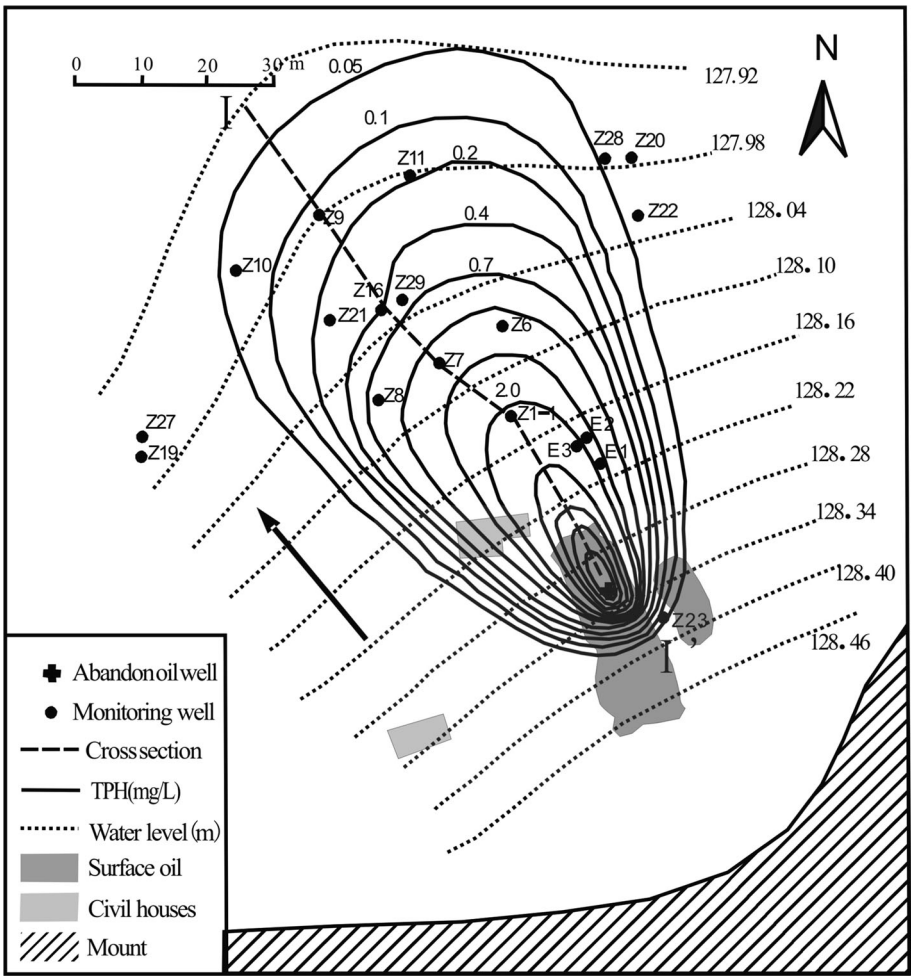

Fig. 2 Map of the groundwater flow regime and the plume of TPH contaminated groundwater

samples for the analysis of ^{14}C in DIC were collected from the wells located along the central line and on the edge of the contamination plume.

Monitoring wells were purged for at least 3–5 well volumes before sampling. Groundwater samples were collected for laboratory analyses once the pH, temperature, electrical conductivity and E_h values had stabilized.

Groundwater samples for TPH analysis were collected in 1-L amber glass bottles and immediately acidified with HCl (to pH < 2) and sealed without a headspace. Temperature, pH, electrical conductivity and E_h were measured on site using a W-23XD multiparameter meter (Japan).

The soil samples were collected from different depths using an auger. After drilling to the target depth, the sample at the tip of the drill was collected quickly. The samples were stored in 60-mL amber glass bottles with no headspace.

Sample analysis

1. Chemical analysis

TPH in groundwater samples was determined by infrared spectral colorimetry (JDS-108U+, China). The sample alkalinity was measured by acid–base titration, and HCO_3^- was determined by phenolphthalein titration.

2. Radiocarbon analysis

The ^{14}C content of the DIC sample aliquots taken from the stable isotope samples was analyzed by Beta Analytic Inc. CO_2 was converted to graphite, and the ^{14}C content of the graphite was analyzed using an accelerator mass spectrometer. The results from these analyses are reported as fractions of modern (pre-1950) C values (values >100 pmc come from samples containing radiocarbon produced during the aboveground testing of nuclear weapons). The precision of the analyses was $\leq 0.01 \times$ modern C values.

3. X-ray diffraction

XRD analysis was performed using a D8 Advance instrument (Bruker, Billerica, MA, USA). The routine operating conditions were 40 kV/40 mA, step scanning at 0.05/30 s in the 2θ range 3–70°.

4.. Microbial community analysis

DNA extraction and PCR amplification

The membranes filtered in the field were sheared using sterile scissors. Total genomic DNA was extracted using the Soil Total DNA Extraction kit (FastDNA® Spin Kit for Soil, MP Biochemicals LLC, Santa Ana, CA, USA). The extracted DNA samples were kept at −20 °C for the subsequent experiments. The variable regions (V3) of 16S rDNA of the microbial community were amplified with the primers GC-F338 and R518. PCR reactions were performed in a Stratagene Mx3000P thermal cycler (Stratagene Laboratories, USA) in a 50-μL reaction volume. The quality of the resulting PCR amplification was confirmed by electrophoresis in 0.8 % (w/v) agarose gels. After staining with ethidium bromide, DNA could be visualized with a UV transilluminator (MiniBIS Pro, DNR).

Denaturing gradient gel electrophoresis (DGGE)

The 220-bp PCR-amplified DNA products were separated by DGGE with the DCode™ universal mutation detection system (Bio-Rad, Hercules, CA, USA). The DGGE image was acquired using the Tannon gel documentation system. PCR product was loaded on to 8 % (w/v) polyacrylamide gels with a denaturing gradient of 30–60 % (100 % denaturant plus 40 % formamide).

Band excision, reamplification and sequencing

DNA bands chosen for sequence analysis were carefully excised from the DGGE gel with a sterile scalpel. For each selected DNA band, only the middle portion of the band was excised and placed into a sterilized microcentrifuge tube. The DNA fragments were recovered from the gel by the EZ Spin Column PAGE Gel DNA Extraction kit (Shanghai Sangon Biological Engineering Technology & Service Co., Ltd.). Two μl of the supernatants were used as templates for the reamplification (as described above). The resulting amplicons were again electrophoresed on a DGGE gel to verify the position of the original band. Bands of DNA were reexcised and treated as described above when necessary. Bands were sequenced by Shanghai Sangon, and sequencing reactions were run on an ABI 3730 apparatus. All sequences were compared with those in the GenBank database (http://www.ncbi.nlm.nih.gov).

Results and discussion

^{14}C isotope evidence for biodegradation of PHC

Background carbon isotopic signatures

The shallow confined aquifer at the contaminated site has a relatively closed structure that is difficult for air and precipitation to infiltrate. The groundwater p_{CO_2} is about 10^{-1} atm, which is much larger than the atmosphere a p_{CO_2} ($10^{-3.5}$ atm), so the impact of atmospheric CO_2 on the groundwater DIC can be ignored.

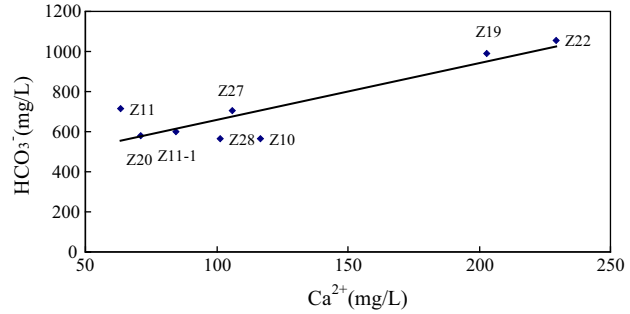

Fig. 3 HCO_3^- concentration plotted against Ca^{2+} concentration for the slightly contaminated groundwater

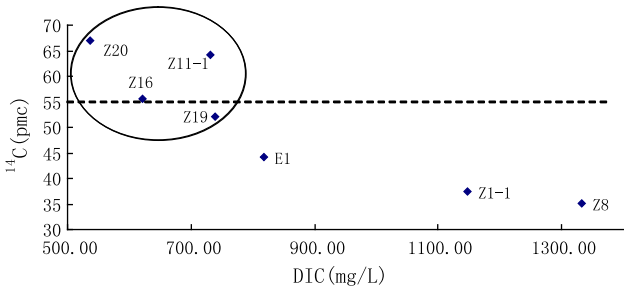

Fig. 4 ^{14}C variations relative to DIC variations in the groundwater dissolved inorganic carbon

The clay layer above the aquifer has a high organic C content of up to 2.224 %, indicating that the aquifer media is rich in organic C and the ^{14}C content of the soil organic carbon is about 110 pmc (Conrad et al. 1997).

According to the X-ray diffraction mineral analysis, the calcite contents of all of the aquifer media were up to approximately 5 %. Low TPH and DIC contents and high contents of electron acceptors in the groundwater suggest that the downgradient groundwater and the plume edge were slightly polluted with PHC. There was a very significant positive correlation between the HCO_3^- and Ca^{2+} contents in the slightly polluted groundwater (Fig. 3), indicating that carbonate was formed by the dissolution of the soil gas CO_2 in the groundwater, increasing the concentrations of both Ca^{2+} and HCO_3^- (see Eq. 1):

$$CaCO_{3(s)} + CO_{2(soil)} + H_2O = Ca^{2+} + 2HCO_3^-. \quad (1)$$

The isotopic composition of the DIC can be calculated using an isotope mass balance equation (Chapelle and Knobel 1986) as shown below in Eq. 2:

$$^{14}C = \frac{\sum_0^i (m_{c_i})(^{14}C_i)}{\sum_0^i (m_{c_i})}, \quad (2)$$

Table 1 Cloning and sequencing results of typical DGGE bands

Sequence number	The highest homology strains in the GenBank database (serial number)	Similarity (%)
1	*Flavobacterium indicum* GPTSA100-9 (NC 017025.1)	88
2	*Arcobacter* sp. *L* (NC 017192.1)	99
	Arcobacter nitrofigilis DSM 7299 (NC 014166.1)	
3	*Pseudomonas fluorescens* SBW25 (NC 012660.1)	98
	Pseudomonas syringae pv. *phaseolicola* 1448A (NC 005773.3)	
	Pseudomonas protegens Pf-5 (NC 004129.6)	
	Pseudomonas syringae pv. *syringae* B728a (NC 007005.1)	
4	*Pseudomonas fluorescens* F113 (NC 016830.1)	98
	Pseudomonas brassicacearum subsp. *brassicacearum* NFM421 (NC 015379.1)	
	Pseudomonas putida W619 (NC 010501.1)	
5	*Dechloromonas aromatica* RCB(NC 007298.1)	97
8	*Paracoccus denitrificans* PD1222 (NC 008687.1)	88
9	*Acidovorax citrulli* AAC00-1 (NC 008752.1)	88
12	*Candidatus Vesicomyosocius okutanii* HA (NC 009465.1)	90
	Methylomicrobium alcaliphilum (NC 016112.1)	
	Methylomicrobium alcaliphilum str. 20Z (FO 082060.1)	
14	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966 (NC 008570.1)	87
16	*Aeromonas salmonicida* subsp. *salmonicida* A449 (NC 009348.1)	100
17	*Neorickettsia risticii* str. *Illinois* (NC 013009.1)	89
	Rickettsia montanensis str. OSU 85-930 (NC 017043.1)	
	Orientia tsutsugamushi str. *Ikeda* (NC 010793.1)	
	Orientia tsutsugamushi str. *Boryong* (NC 009488.1)	

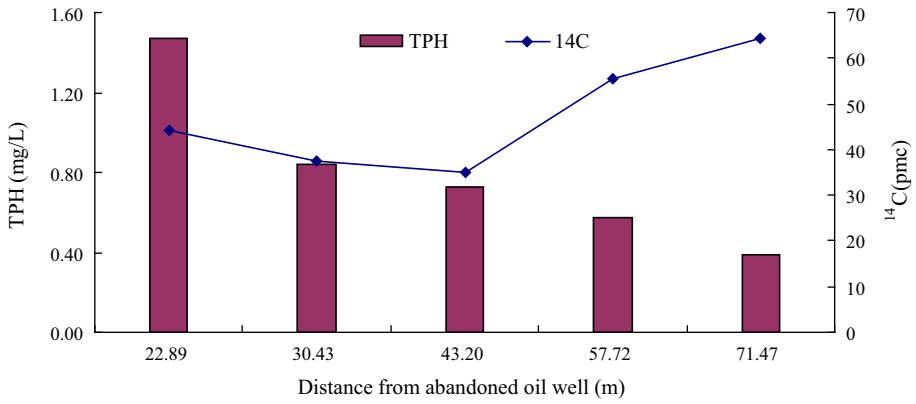

Fig. 5 Variations in the TPH and ^{14}C values along the groundwater flow direction

where m_{ci} is the molality of the added DIC from the *i*th source and $^{14}C_i$ is the ^{14}C of the added DIC from the *i*th source.

The average ^{14}C in the soil organic carbon was about 110 pmc, and the ^{14}C of the carbonate was about 0 ‰ (Clark and Fritz 1997). The ^{14}C value in the groundwater DIC was calculated to be about 55 pmc using Eq. 2, based on equal mixing of the soil gas CO_2 and the carbonate. The calculated ^{14}C value is in good agreement with the ^{14}C value found in the slightly polluted groundwater downgradient of the contaminant plume (in wells Z16 and Z19).

Evidence for biodegradation processes

As discussed above, the ^{14}C values in the uncontaminated groundwater samples were about 55 pmc, and the wells downgradient and at the edge of the contaminated plume had similar ^{14}C values. The ^{14}C values in water from the wells upgradient of the plume (E1, Z1-1 and Z8) were clearly lower than the ^{14}C values downgradient and at the edge of the plume (Fig. 4). There are two possible sources of the low DIC ^{14}C value, the biodegradation of PHC and carbonate dissolution, but the relatively low concentration of Ca^{2+} and the positive saturation index for carbonate in these wells showed that carbonate dissolution was not the main source of DIC in the contaminated groundwater. Therefore, the relatively low ^{14}C values in the contaminated groundwater were caused by the biodegradation of PHC (Table 1).

The spatial variations in the ^{14}C values further confirmed the analysis described above, in that the ^{14}C values indicated that relatively modern C was present (Fig. 5) along the TPH plume from the contaminated source along the downgradient, with decreasing TPH and DIC concentrations. This indicated that the biodegradation of the PHC contributed more DIC with low ^{14}C values to the groundwater upgradient of the contaminated plume than downgradient of the plume, which demonstrated the occurrence of PHC biodegradation processes.

Microbial community structure as evidence for biodegradation of PHC

The results of PCR–DGGE of groundwater samples in the contaminated site are shown in Fig. 6, in which 12 typical DNA electrophoresis bands was chosen to be cloned and sequenced, and the sequencing results were compared with known sequences in GenBank.

The sequence of band 5 was most similar to *Dechloromonas aromatica* RCB, with 97 % homology, which was found in waters and sediments, and had the ability to biodegrade aromatic compounds, such as benzene and toluene. This strain was also the only organism in pure culture that was capable of anaerobically oxidizing benzene (Salinero et al. 2009; Coates et al. 2001). The sequence of band 4 had the highest homology with *Pseudomonas putida*

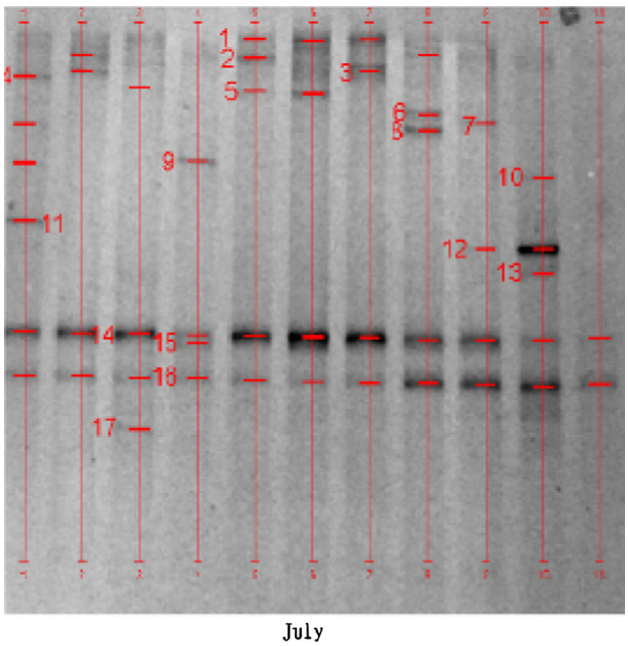

Fig. 6 DGGE of DNA extracted from groundwater contaminated by petroleum (Nos. 1–11 are samples S1, E1, E2, Z1-1, Z6, Z8, Z10, Z16, Z19, Z20 and Z22, respectively)

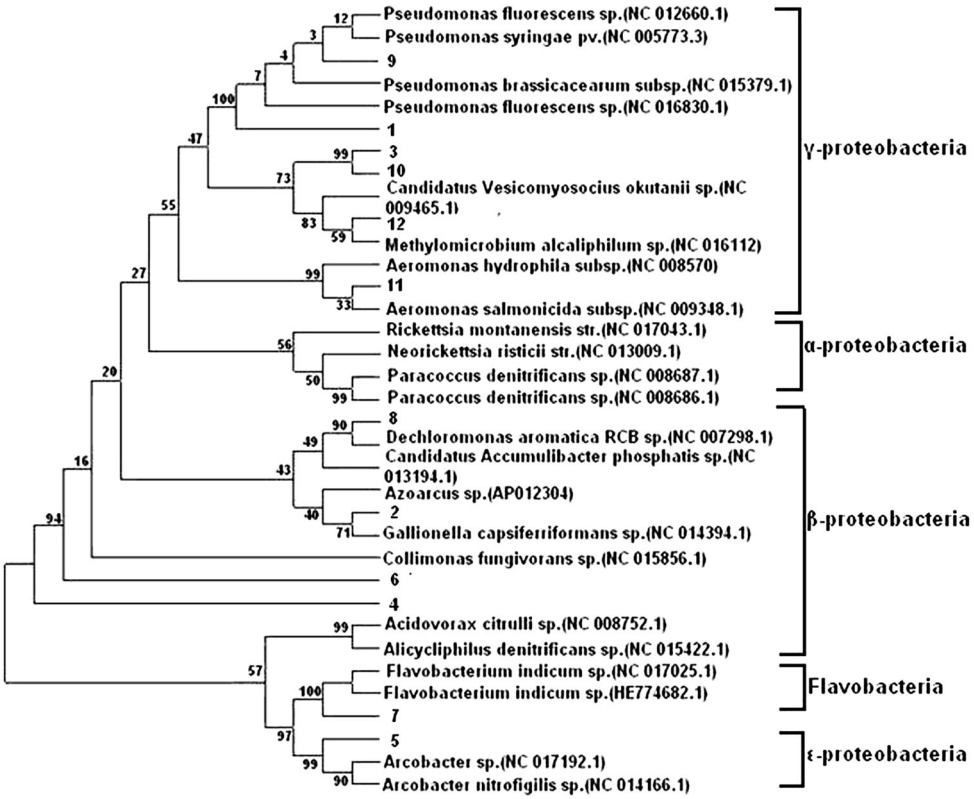

Fig. 7 16S rRNA (V3) gene phylogenetic tree of typical DGGE bands representing corresponding microorganisms

(up to 98 %), a gram-negative, rod-shaped bacterium, capable of biodegradation of many organic substances such as toluene, naphthalene and phenol, which has been widely used in the processes of PHC biodegradation (Marcus 2003; Kowalski 2002). The sequence of band 3 was most similar to *Pseudomonas protegens* Pf-5, with homology of up to 98 %, which could biodegrade polycyclic aromatic hydrocarbons, including fluorene, anthracene, phenanthrene, pyrene and benzopyrene (Kanehisa Laboratories 2004). The sequence of band 2 had the highest homology with *Arcobacter nitrofigilis* DSM 7299, which was capable of nitrogen fixation (Fera et al. 2004) and could promote the PHC biodegradation reactions. The analysis of microbial communities in the groundwater demonstrated the occurrence of biodegradation processes in the contaminated site (Fig. 7).

Conclusions

Stable carbon isotopes alone can yield ambiguous results when identifying the existence of PHC biodegradation processes, as there can be overlap between the stable isotope ratios of hydrocarbons and of indigenous plants, and the ratios can be impacted by methanogenesis. This study combined analysis of radiocarbon and microbial community structures to demonstrate *in situ* biodegradation of PHC in a contaminated site. The results indicated that the radiocarbon measurement could identify the biodegradation of PHC, and method associated with PCR analysis allowed a better understanding of biogeochemical conditions.

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