Succession of bacterial community along with the removal of heavy crude oil pollutants by multiple biostimulation treatments in the Yellow River Delta, China

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Abstract

Multiple biostimulation treatments were applied to enhance the removal of heavy crude oil pollutants in the saline soil of Yellow River Delta. Changes of the soil bacterial community were monitored using the terminal restriction fragment length polymorphism (T-RFLP) and clone library analyses. The 140-day microcosm experiments showed that low C:N:P ratio, high availability of surfactant and addition of bulking agent significantly enhanced the performance, leading to the highest total petroleum hydrocarbon removal. Meanwhile, the bacterial community was remarkably changed by the multiple biostimulation treatments, with the Deltaproteobacteria, Firmicutes, Actinobacteria, Acidobacteria and Planctomycetes being inhibited and the Alpha- and Beta-proteobacteria and some unknown Gammaproteobacteria bacteria being enriched. In addition, different hydrocarbon-degraders came to power in the following turn. At the first stage, the *Alcanivorax*-related Gammaproteobacteria bacteria dominated in the biostimulated soil and contributed mainly to the biodegradation of easily degradable portion of the heavy crude oil. Then the bacteria belonging to Alphaproteobacteria, followed by bacteria belonging to Candidate division OD1, became the dominant oil-degraders to degrade the remaining recalcitrant constituents of the heavy crude oil.

Key words: heavy crude oil contamination; biostimulation; bacterial community; T-RFLP; Yellow River Delta; saline soil

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Introduction

Bioremediation has been recognized as a cost-effective and environment-friendly approach for cleanup of the petroleum pollutants (Jiménez et al., 2006; Venosa et al., 1996) by stimulating the indigenous soil microorganisms. In fact, hydrocarbon-degrading bacteria have been found ubiquitous in soil (Leahy and Colwell, 1990), forming different microbial consortia to degrade crude oil components.

These bacteria can be stimulated when provided favorable conditions (Evans et al., 2004; Kaplan and Kitts, 2004; Macnaughton et al., 1999; Ogino et al., 2001; Röling et al., 2002; Viñas et al., 2005), such as amending nitrogen and phosphorous (Bento et al., 2005; Jiménez et al., 2006), adding surfactant or biosurfactant (Rahman et al., 2002) as well as supplementing bulking agents like wheat straw, hay and sawdust (Rahman et al., 2002; Namkoong et al., 2002; Rhykerd et al., 1999). As a consequence, an improved

degradation of hydrocarbon pollutants in the soil environment can be achieved, resulting in significant dynamics of microbial community structure. For example, the bacterial community of oil contamination soil showed greater changes by the addition of inorganic compounds (phosphorus and K, Ca, and Mg) (Evans et al., 2004). When the surfactant was added to a hydrocarbon-contaminated soil, original-dominant Gram-positive *Rhodococcus* and *Nocardia* populations were replaced by Gram-negative *Pseudomonas* and *Alcaligenes* populations (Colores et al., 2000). In fact, *Pseudomonas* spp. are commonly detected in various hydrocarbon-contaminated environments using culture-dependent (Belhaj et al., 2002; Bhattacharya et al., 2003) and culture-independent (Kaplan and Kitts, 2004; Margesin et al., 2003) approaches.

Whilst intensive studies have been conducted concerning the changes of bacterial community with the use of different individual biostimulation approaches, little is known so far on how the combination of multiple biostimulation may affect the total petroleum hydrocarbon (TPH) removal and the bacterial community. In addition,

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the bacterial community may vary significantly in different geographic regions. The Yellow River Delta, as one of the youngest and fastest-warming estuary deltas of the world and with, the second largest oil field of China (Shengli Oilfield) located, has witnessed frequent heavy crude oil pollution in the past decades. Thus, it is significant to understand how the bacterial community in the special saline soil in the Yellow River Delta may response to heavy crude oil contamination so that effective control of oil contamination can be achieved.

Microcosm experiments were conducted for the saline soil in the Yellow River Delta. Responses of the saline soil bacterial community to the heavy crude oil from Shengli Oil Field and to a combination of several biostimulation approaches were investigated by comparing the bacterial community of the blank soil, the control microcosm with oil pollution and the microcosm with combination of optimal biostimulation parameters. Dynamics of the bacterial community were examined using the widely-used terminal restriction fragment length polymorphism (T-RFLP) technique (Kaplan and Kitts, 2004; Brito et al., 2006; Katsivela et al., 2005; Wu et al., 2002, 2006) in conjunction with clone library analysis.

1 Materials and methods

1.1 Characteristics of the soil and heavy crude oil

The non-contaminated background soil was sampled from the Yellow River Delta in Dongying, Shangdong Province of China. Surface litter was removed and soil was collected from the upper soil layer (0–20 cm). The collected soil was then sieved through 2 mm sieve to remove large roots, macrofauna and stones. The soil samples were characterized following the standard methods (Lu, 2000). The composition of the soil was: sand 20%; silt 43%; clay 27%; soluble salt (2.5 ± 0.2) g/kg; organic matter (11.5 ± 1.1) g/kg; available nitrogen (42.8 ± 3.1) g/kg; available phosphorus (2.2 ± 0.4) g/kg; and pH was 7.8 ± 0.2. The typical heavy crude oil from the Shengli Oilfield was used, which contains approximately 39.05% saturates, 30.90% aromatics, 23.18% resins and 6.87% asphaltenes.

1.2 Microcosm experiment

Homogenous mixture of the heavy crude oil and the background non-contaminated soil was filled, 10 kg each, into 4 reactors (size: 35 cm length × 30 cm width × 15 cm height) to generate 4 aerobic microcosms (total petroleum hydrocarbon (TPH) 37.85 g/kg soil). In each microcosm, a 5 cm-thick small stone layer combined with steel-wire screen and porous plate were assembled for air supply. A control microcosm (RC) was set to investigate the response of the saline soil bacterial community to heavy crude oil contamination and the background TPH removal efficiency without biostimulation, whereas the other three microcosms were used to explore that under various biostimulation situation. All microcosms were incubated at $(20 \pm 5)°C$ for 140 days and tilled periodically to ensure sufficient aerobiosis. Distilled water was sprayed

to maintain the soil moisture at $20\% \pm 3\%$. Soils were sampled at the center and four corners of each microcosm and mixed as one sample at day 10, 30, 70, 100 and 140 after the experiment started. The mixed soil samples were then subjected to the petroleum hydrocarbon analysis and to the DNA extraction for T-RFLP and clone library analyses.

1.3 DNA extraction and PCR amplification

Soil samples from microcosm R1 (the highest TPH removal efficiency achieved among three microcosms) and RC at day 0, 10, 30, 70, 100 and 140 were used to extract DNA according to a previous reported procedure (Henckel et al., 1999; Moré et al., 1994). The bacterial 16S rRNA gene fragment was amplified in a 50 μL reaction containing 5 μL 10× PCR-buffer, 15 mmol/L MgCl₂ (Takara, Dalian, China), 200 μmol/L dNTP (Takara), 10 pmol of each primer (Applied Biosystems, Shanghai, China) (8F: 5′-AGAGTTTGATCCTGGCTCAG-3′; 1492R: 5′-GGTTACCTTGTTACGACTT-3′ (Dojka et al., 1998; Park et al., 2006), 1.5 U *Taq* DNA-polymerase (Takara) and approximate 10 ng template DNA. PCR was performed in a thermocycler (PTC-200, MJ Research Inc., Watertown, USA) with the thermal profile: initial denaturing 5 min at 94°C, followed by 30 cycles of denaturing (94°C for 1 min); annealing (51°C for 45 sec) and elongation (72°C for 2 min). Both the unlabeled and 6-carboxyfluorescein (6-FAM) labeled 8F primers were used to amplify the DNA fragment for clone library and T-RFLP analyses, respectively. The DNA amplicons were purified with the QIAquick PCR Purification Kit (Qiagen Co., Shanghai, China).

1.4 T-RFLP analysis

Ten microliter purified DNA amplicon was digested in a 20-μL reaction for 6 hr at 37°C with 20 U of *Rsa*I (New England Biolabs, Beverly, USA) according to the manufacturer's instruction, followed by a desalting step (Fedi et al., 2005). The purified digested DNA was mixed with 12 μL Hi-Di formamide and 0.5 μL of DNA fragment length internal standard (GeneScan Liz-500, Applied Biosystem, IL, USA), denatured at 95°C for 5 min, followed by immediately snap-cooling on ice. The "Genescan" analysis was then conducted in a capillary electrophoresis system (ABI 3130 Genetic Analyzer, Applied Biosystems) according to the manufacturer's instruction. Fragment separation data were collected with ABI 3130 Collection (version 2.7) and GeneMapper Analysis Software (version 3.7). The peaks with the terminal restriction fragment (T-RF) of 50–800 bp were regarded as effective peaks and the relative peak area of every single T-RF was calculated by dividing the individual T-RF peak area by the total area of all the peaks.

1.5 Statistical analysis of T-RFLP profiles

Temporal changes in the bacterial community of microcosms RC and R1 were tracked by the T-RFLP analysis during the entire experiment period. Eleven T-RFLP profiles (the background soil, BS, plus soils from both RC

and R1 microcosms collected at five different time points), each with 41 unique fragments, were evaluated and visualized by Multidimensional Scaling (MDS) analysis using software SPSS v15.0 based on the calculation of Euclidean distances.

1.6 Cloning, screening and sequencing

Two bacterial 16S rRNA gene clone libraries were constructed for the background soil (BS-000) and the soil sampled at day 140 from microcosm R1 (R1-140), respectively. From the BS-000 and R1-140 clone libraries 182 and 191 positive clones were picked up, respectively. The clones were screened by both restriction fragment length polymorphism (RFLP) and T-RFLP analyses with the *Rsa*I and *Hha*I (New England Biolabs, Beverly, MA, USA) as restriction enzymes. Then the clones were classified into different groups according to the T-RFLP and RFLP patterns. Representative clones from different T-RF groups were randomly chosen and sequenced. After the Chimera Check on the Ribosomal Database Project, the clone sequences were aligned and phylogenetic trees were constructed by the neighbor-joining method with the Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura et al., 2007).

1.7 Chemical analyses

The TPH in 20 g soil sample was Soxhlet extracted three times with 100 mL of chloroform and evaporated at room temperature. The percentage of TPH removal was calculated based on the weight difference. The TPH extracts were fractionated into saturates, aromatics, resins and asphaltenes by liquid-solid chromatography (Chaillan et al., 2004). The saturate and aromatic fractions were analyzed using gas chromatography coupled with mass spectrometry (GC-MS, Thermo-Finnigan Trace-DSQ, USA) with the HP-5MS column (60 m × 0.2 mm × 0.25 μm) and helium as carrier gas at a flow rate of 1 mL/min. The temperatures of the injector and the detector were 300 and 280°C, respectively. The temperature program for the analysis of saturate fraction was: 50°C for 1 min, increase by 20°C/min to 120°C, increase by 4°C/min to 250°C, increase by 3°C/min to 310°C, hold for 30 min. The temperature program for the analysis of aromatic fraction was: 50°C for 1 min, increase by 15°C/min to 120°C, increase by 3°C/min to 300°C, hold for 35 min.

1.8 Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers: EU327998 to EU328106.

2 Results

2.1 Total petroleum hydrocarbon (TPH) removal of different microcosms

After 140-day operation, a TPH removal efficiency of 9.22% was achieved in the control microcosm. The biostimulation treatments remarkably increased TPH removal.

Table 1 Microcosms experiments

Microcosms	Operation parameter			TPH removal efficiency (%)
	C:N:P (*m/m/m*)	Surfactant (mL/kg dry soil)	Soil bulking agent (*W/W*)	
R1	200:10:1	1.5	5% sawdust	30.80
R2	100:10:1	0	w/a	15.29
R3	40:7:1	0.5	5% straw	22.94
RC	w/a	w/a	w/a	9.22

N: added in $(NH_4)_2SO_4$; P: added in KH_2PO_4; surfactant: Tween 80; w/a: without addition.
RC: control microcosm ; TPH: total petroleum hydrocarbon.

R1 microcosm (C:N:P: 200:10:1, *m/m/m*; Tween 80: 1.5 mL/kg dry soil; bulking agent: 5% sawdust) exhibited the highest TPH removal efficiency of 30.80% (Table 1). Since no significant changes were observed in the diagnostic parameters (Table 2) as well as the saturates and aromatics fingerprints (Fig. 1) in microcosm RC, the limited decrease of TPH concentration in microcosm RC could be attributed to abiotic processes such as evaporation of low-molecular-weight hydrocarbons. In contrast, both the diagnostic parameters, including pristane to phytane ratio (Pr/Ph) and the $\sum nC_{10-14}(\%)$ and the $\sum nC_{21-27}(\%)$ (Seklemova et al., 2001), as well as the saturates and aromatics fingerprints changed remarkably in microcosm R1 (Table 2, Fig. 1), suggesting the microbial activity in TPH degradation was significantly enhanced in microcosm R1. Since R1 showed the highest TPH removal, the soil in R1 was used to analyze the bacterial community structure by both T-RFLP and clone library analyses.

### 2.2 Comparisons between the two clone libraries

After screening by T-RFLP and RFLP analyses, 58 representative clones from 182 clones in library BS-000 and 50 representative clones from 191 clones in library R1-140 were sequenced and named by "B+number" and "Y+number", respectively. Bacteria belonging to the phylum Proteobacteria were predominant in both BS-000 and R1-140 libraries, accounting for 58.79% and 57.59% of the total clones, respectively (Table 3), followed by the clones aligned to the phylum Bacteroidetes (CFB group) with the relative abundance of 32.97% and 41.36% in BS-000 and R1-140. Four other bacterial phyla, Firmicutes (2.75%), Actinobacteria (2.20%), Acidobacteria (0.55%) and Planctomycetes (0.55%), were only retrieved from the BS-000 library, suggesting strong negative impacts of the oil contamination and/or the multiple biostimulation treatments on these populations. In addition, two clones from both library BS-000 and R1-140 were identified as belonging to the Candidate division OD1.

Within the phylum Proteobacteria, clones assigned to Gammaproteobacteria dominated in both clone libraries (Table 3), among which bacteria closely related to *Marinobacter* (T-RF 168 bp) and *Alcanivorax* (T-RFs of 463 and 468 bp) were predominant in the background soil (library BS-000) (Fig. 2). By comparison, the library R1-140 was dominated by the clones belonging to *Marinobacter* (168 bp) and T-RF 774 bp bacteria which did not have pure-cultured relatives.

Table 2   Total petroleum hydrocarbon (TPH) removal and biodegradation indicators in microcosms RC and R1

Microcosms	Time (day)	TPH removal efficiency (%)	Pr/Ph	$\sum n\mathrm{C}_{10-14}$(%)	$\sum n\mathrm{C}_{21-27}$(%)
RC	0	0	0.47	0.76	4.6
	10	1.20			
	30	4.68			
	70	7.94			
	100	8.77			
	140	9.22	0.46	0.72	4.7
R1	0	0	0.47	0.76	4.6
	10	4.93			
	30	11.31			
	70	15.15			
	100	24.79			
	140	30.80	0.38	0	1.7

Pr/Ph: ratio of pristane and phytane; $\sum n\mathrm{C}_{10-14}$: percentage of hydrocarbons with carbon number from 10 to 14; $\sum n\mathrm{C}_{21-27}$: percentage of hydrocarbons with carbon number from 21 to 27.
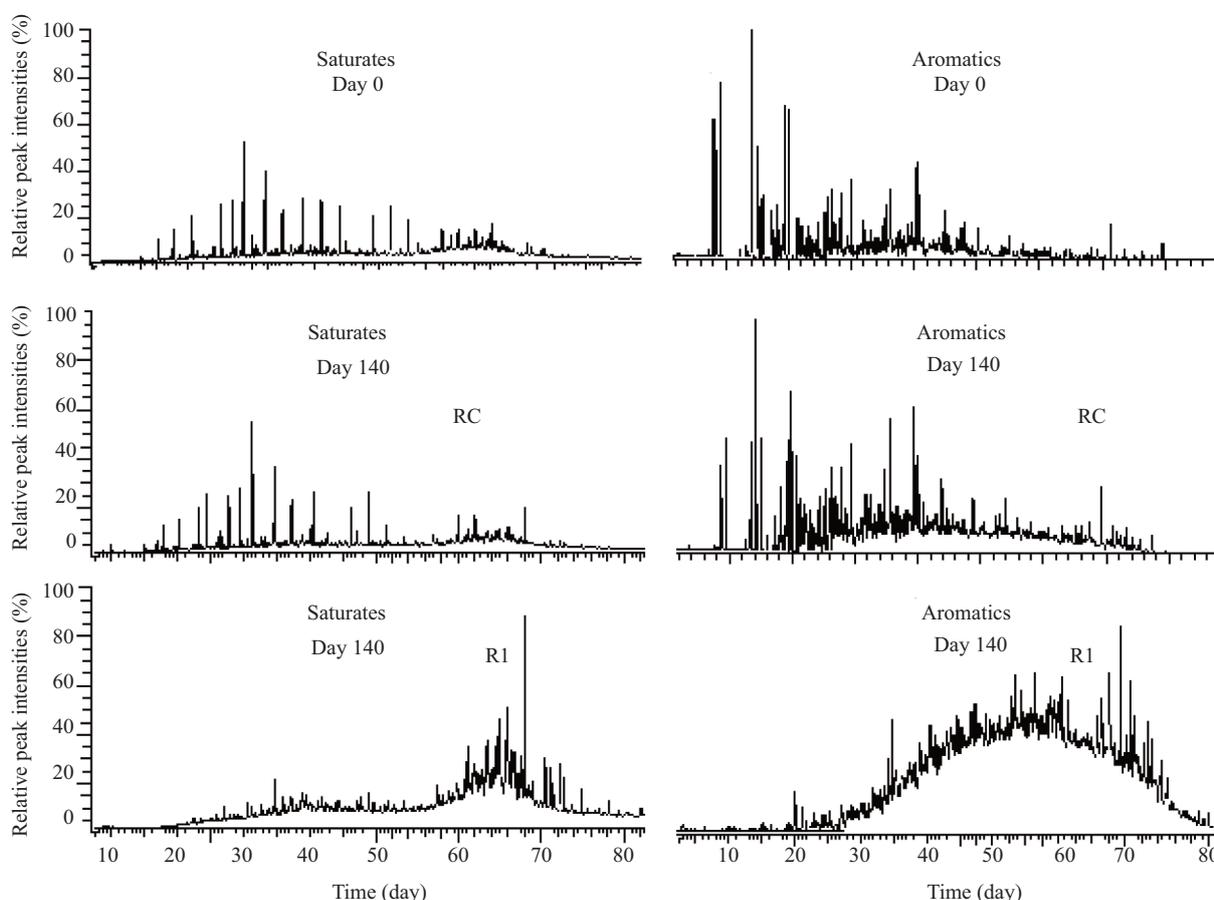
Fig. 1 GC-MS fingerprints of the representative saturates and aromatics fractions of the heavy crude oil from microcosm RC representing the Yellow River Delta saline soil newly subjected to heavy crude oil pollution, and microcosm R1 representing the Yellow River Delta saline soil subjected to heavy crude oil pollution and immediate multiple biostimulation treatments, at day 0 and day 140.

Number of clones assigned to Alphaproteobacteria in library R1-140 was two times of that in library BS-000. Clones with T-RF of 768 bp, the Rhodobacteraceae species, were only retrieved from library R1-140. However, some clones with T-RFs of 115 and 417 bp, which are closely related to species in genera *Erythrobacter* or *Sphingomonas*, were detected in both libraries (Fig. 3). The Deltaproteobacteria clones were only retrieved from library BS-000, which were closely related to the genus *Desulfuromonas* and the uncultured environmental clones (Fig. 3). In contrast, clones belonging to Betaproteobacte-

ria were only detected in library R1-140, all of which were *Alcaligenes* relatives.

Clones assigned to the phylum Bacteroidetes (CFB group) were highly diverse (Fig. 4). Clones with T-RFs of 305 and 309 bp dominated in library BS-000 while clones with T-RFs of 230, 309 and 315 bp were dominant in library R1-140. Bacteria with T-RF 305 bp, were classified to the orders Flavobacteriales and Sphingobacteriales, with no pure-cultured relatives. Bacteria with T-RF of 309 bp were closely related to species in genera *Salegentibacter* and *Owenweeksia* in Flavobacteriales. Clones with T-RF

Table 3   Classification of clones retrieved from libraries of BS-000 and R1-140

Phylotype	BS-000 library			R1-140 library		
	OTU No.	Clone No.	Percentage (%)	OTU No.	Clone No.	Percentage (%)
Proteobacteria	30	107	58.79	23	110	57.59
Alphaproteobacteria	5	7	3.85	6	16	8.38
Betaproteobacteria	0	0	0	1	6	3.14
Gammaproteobacteria	19	82	45.05	16	88	46.07
Deltaproteobacteria	6	18	9.89	0	0	0
Bacteroidetes	17	60	32.97	13	79	41.36
Actinobacteria	2	4	2.20	0	0	0
Acidobacteria	1	1	0.55	0	0	0
Planctomycetes	1	1	0.55	0	0	0
Firmicutes	2	5	2.75	0	0	0
Verrucomicrobia	0	0	0	1	1	0.52
Candidate division OD1	1	1	0.55	1	1	0.52
Unclassified	1	3	1.65	0	0	0
Total	55	182	100	38	191	100
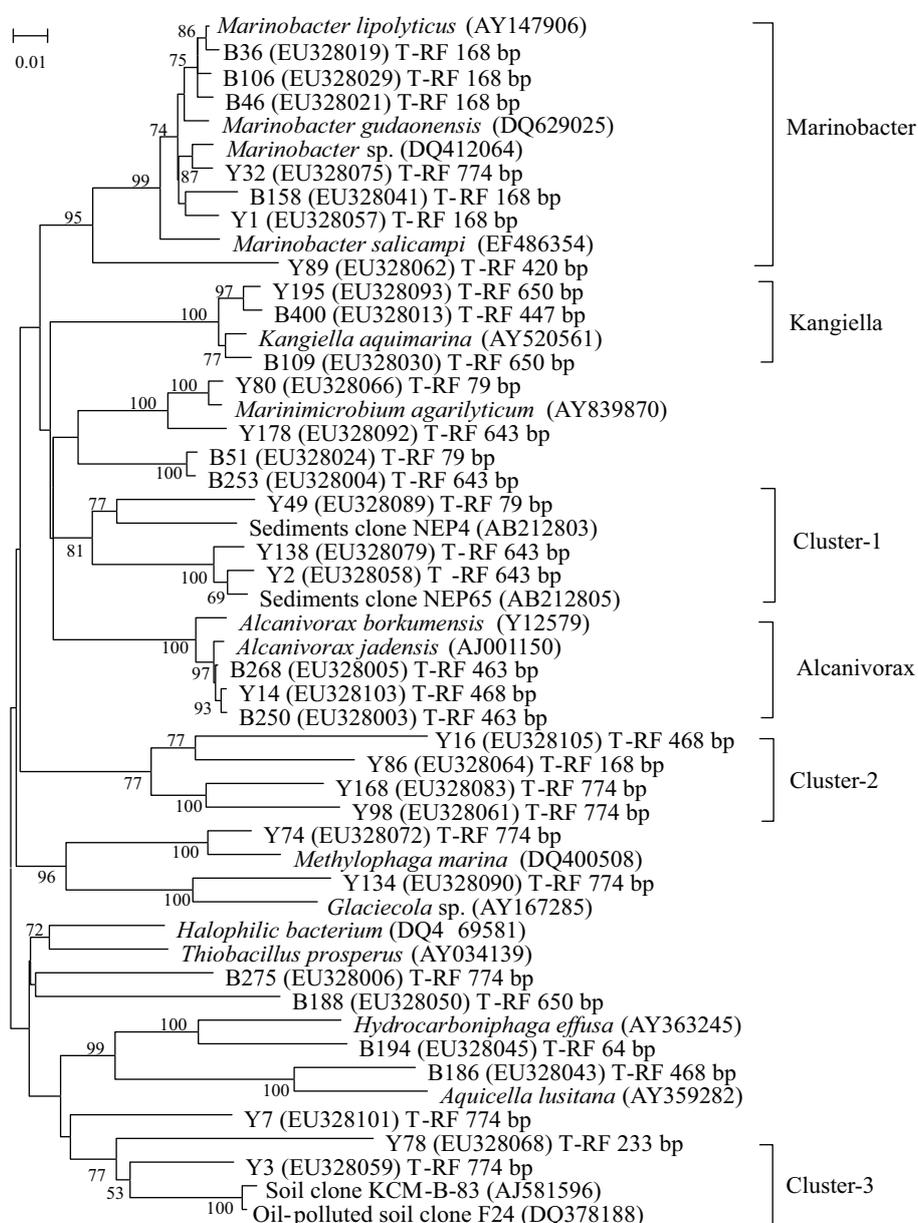
Fig. 2   Phylogenetic relationship of the nearly-complete 16S rRNA gene sequences of bacteria belonging to the class Gammaproteobacteria recovered from clone libraries of BS-000 and R1-140. The clones from library BS-000 and library R1-140 were named after B and Y, respectively. The phylogenetic tree was constructed using the neighbor-joining algorithm, with the Kimura two parameter correction factor. Bootstrap values higher than 50% are shown at the nodes. The scale bar represents substitutions per nucleotide position. GenBank accession numbers are shown in brackets, and the length of the representative T-RF is listed in the end of each clone. Only one sequenced clone is shown in the phylogenetic tree when a series of clones showed high sequence similarity (above 99%) and shared the same T-RF length.
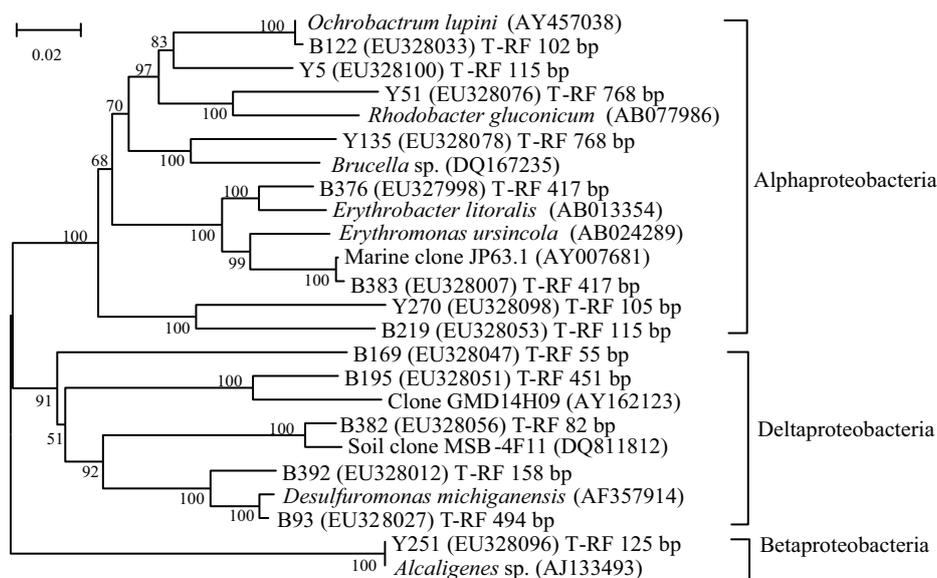
Fig. 3 Phylogenetic relationship of the nearly-complete 16S rRNA gene sequences of bacteria belonging to the classes of Alpha-, Beta-, and Deltaproteobacteria recovered from clone libraries of BS-000 and R1-140. The phylogenetic tree was constructed as described in Fig. 2. The descriptions of other items in the tree are the same as those in Fig. 2.

of 315 bp exclusively formed a cluster highly close to *Salegentibacter salegens* (95%–97%) and *Salegentibacter flavus* (95%–97%). Clones with T-RF of 230 bp were closely related to uncultured Cytophaga clones in the order Sphingobacteriales.

2.3 Temporal change of the bacterial community

During the 140 days operation, the bacterial community changed significantly. T-RFLP analysis reveals a slight difference in the bacterial community of the control microcosm (RC) and the BS soil (Fig. 5a). However, significant changes in bacterial community were observed in the R1 microcosm as a result of multiple biostimulations. As shown in Fig. 5b, the abundance of Gammaproteobacteria species increased immediately from 60% to 68% and remained at that level until day 70, then decreased dramatically to 29% at day 100 and to 12% at day 140. In contrast, the bacteria belonging to Alphaproteobacteria, Bacteroidetes and Candidate division OD1 (T-RF of 429 bp) increased from day 70 to day 140. The Betaproteobacteria was also enriched at the late phase of the experiment (Fig. 5b). This observation was also supported by MDS analysis. Within the first 10 days, bacterial community in both the RC and R1 microcosms differed considerably from that in the BS. After that, the microbial community distinctly formed a very close cluster in the microcosm RC during the whole operation period (Fig. 6), suggesting that no remarkable temporal dynamics occurred in the RC bacterial community. The 5 T-RFLP profiles were widely scattered in the MDS plot in case of the microcosm R1, indicating the significant dynamics of bacterial community in microcosm R1 over time.

2.4 Succession of the dominant species

At the lower taxa, the dominant bacteria witnessed much remarkable changes with time, suggesting an obvious succession of bacterial species.

Among the Gammaproteobacteria, the dominant *Marinobacter*- (168 bp) and *Alcanivorax*-relatives (463 bp) accounted for over 20% in BS (Fig. 7a). In the microcosm RC with heavy crude oil pollution, *Alcanivorax*-relatives increased to 36% at day 30 and returned to the background level at day 100, while *Marinobacter*-relatives remained unchange within the first 30 days, then decreased to 14% at day 70 and finally recovered to the background level at day 140. In microcosm R1, *Marinobacter*-relatives decreased from 21.2% to 2.8% and *Alcanivorax*-relatives increased from 20.8% to over 60% within the first 70 days, followed by a steep decrease to 1.5% at day 140 (Fig. 7b).

For the bacteria belonging to Alphaproteobacteria and Bacteroidetes, not remarkable differences in abundance were found between the background (BS) and the RC soils (Fig. 7c and Fig. 7e), suggesting the strong resistance of these bacteria to the heavy crude oil pollution. In contrast, in R1 the Alphaproteobacteria increased dramatically from 3% to 20%, attributed to the enrichment of some *Erythrobacter*- or *Sphingomonas*-relatives (T-RF 115 bp or 417 bp) and the Rhodobacteraceae species (T-RF 768 bp) (Fig. 7d). Similarly, a distinct shift was also observed in the Bacteroidetes community: bacteria belonging to Bacteroidetes drop to about 6% within the first 70 days, and then rose rapidly to 36% at day 140 because of the remarkable growth of bacteria related to the species in genera *Salegentibacter* and *Owenweeksia* in Flavobacteriales (T-RFs of 309 and 315 bp) (Fig. 7f).

3 Discussion

The much higher TPH reduction (Table 2) and the remarkable change of hydrocarbon composition in microcosm R1 (Fig. 1) indicate that microorganisms played a critical role in the crude oil degradation in saline soil.
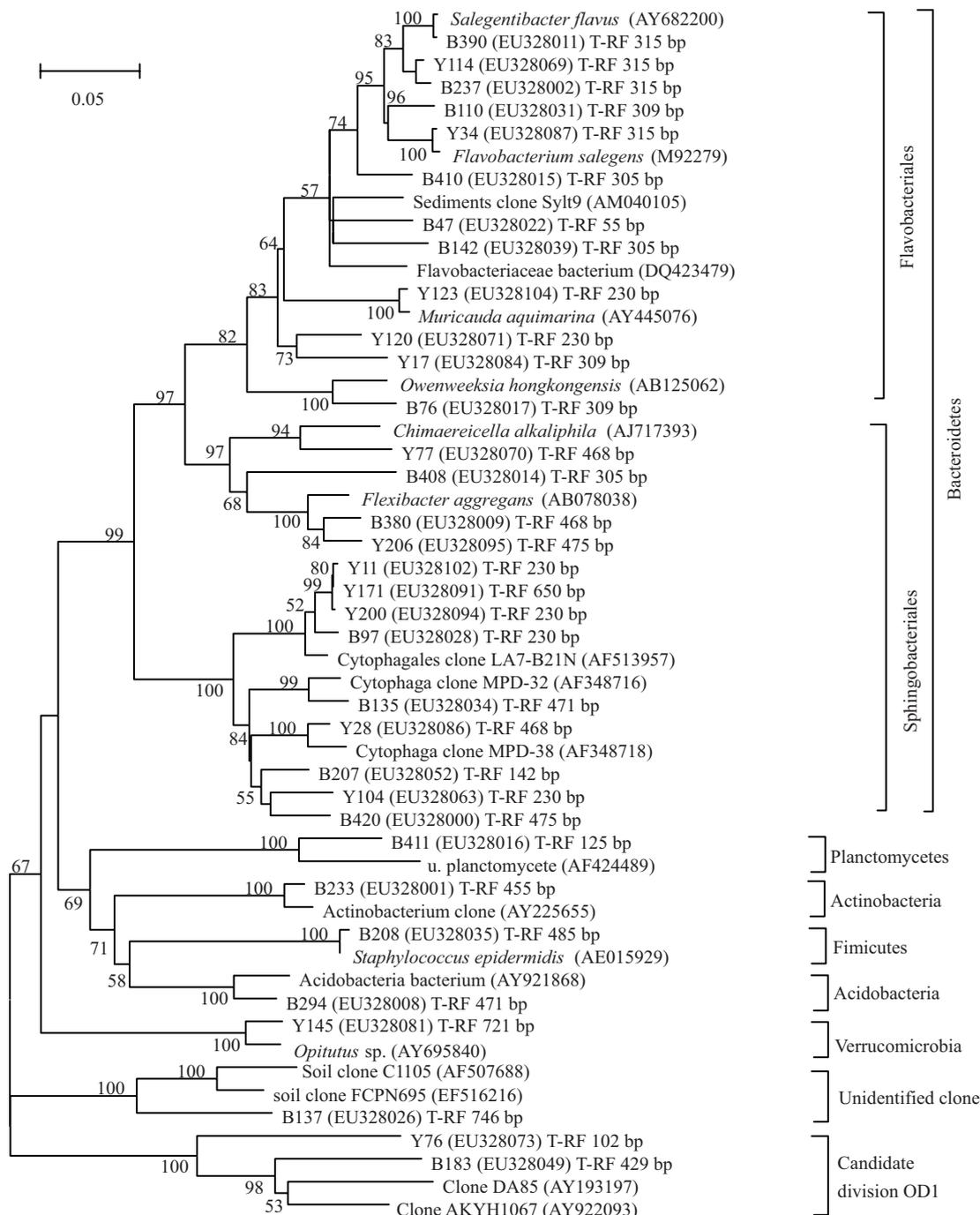
Fig. 4 Phylogenetic relationship of the nearly-complete 16S rRNA gene sequences of bacteria belonging to the Bacteroidetes and other phyla recovered from the clone libraries of BS-000 and R1-140. The phylogenetic tree was constructed as described in Fig. 2. The descriptions of other items in the tree are the same as those in Fig. 2.

The Yellow River Delta soil bacterial community responded differently to heavy crude oil and the multiple biostimulation treatments. According to the MDS plot and the T-RFLP analysis, the dominant bacteria in the Yellow River Delta saline soil were highly resistant and resilient to heavy crude oil contamination. The biostimulation treatments lead to enrichment of some bacteria whilst cause inhibition to others, as is demonstrated by the obvious succession of bacterial community in microcosm R1 (Figs. 5, 6 and 7).

Among all the potential petroleum hydrocarbon degraders, the *Alcanivorax*-relatives with typical T-RF of

463 bp dominated in the Yellow River Delta saline soil. Their dominance was less affected by the heavy crude oil pollution, but significantly altered by biostimulation treatment. Species in the genus *Alcanivorax* were known to degrade oil hydrocarbons and grow on many saturated petroleum fraction constituents (Golyshin et al., 2003; Hara et al., 2003; Liu and Shao, 2005; Yakimov et al., 1998). The drastic increase of the *Alcanivorax*-relatives in microcosm R1 during the first 70 days indicates that these bacteria, which played a key role in the cleanup of hydrocarbons, were effectively stimulated by the multiple biostimulation treatments. This is consistent with the lit-
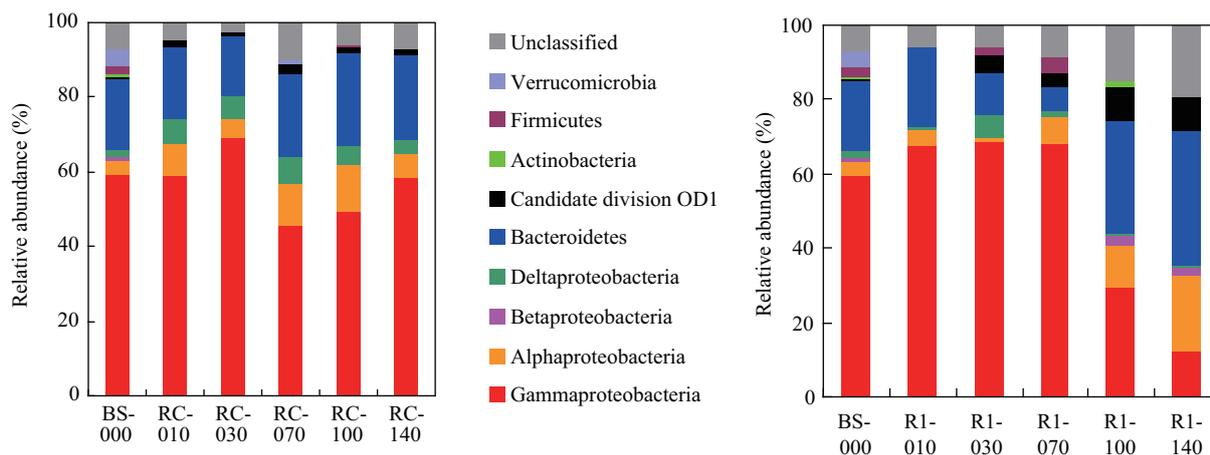
Fig. 5 Dynamics of the relative abundance of bacteria affiliated to different phyla calculated from the T-RFLP profiles of microcosm RC (a) and microcosm R1 (b). Each sample is labeled with name of the soil followed by the day of sampling, for example, BS-000 represents the background non-contaminated Yellow River Delta saline soil sample, R1-010, RC-010 represent to the samples from R1 and RC microcosms at day 10.
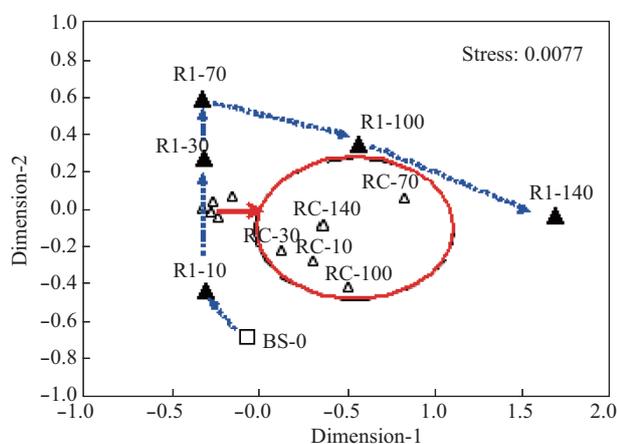
Fig. 6 Multidimensional Scaling Analysis of the bacterial community T-RFLP profiles. The stress was calculated to be 0.0077, which indicates an ideal two dimensional plot of the MDS analysis. The horizontal abscissa is marked by the name of the soil followed by the day of sampling as described in Fig. 5. The RC samples were clustered together, as illustrated by the arrow and the dashed ellipse.

erature that *Alcanivorax* bacteria were always the first to bloom in oil-contaminated soils (Kasai et al., 2002).

Along with the decrease of *Alcanivorax*-relatives from day 70, the continued TPH removal should be attributed to the increase of the bacteria belonging to Alphaproteobacteria and Candidate division OD1, which started to increase at day 30 and day 70, respectively. Species in the genera of *Sphingomonas* and *Ochrobactrum* in Alphaproteobacteria have been reported being able to degrade recalcitrant PAHs (Brito et al., 2006), alkane hydrocarbons (Leys et al., 2004), halobenzoates (Song et al., 2000), and pristane (Brito et al., 2006). The Candidate division OD1 bacteria (T-RF of 429 bp) detected in this study have also been frequently found in hydrocarbon-contaminated environments (Dojka et al., 1998; Harris et al., 2004). These bacteria kept increasing and became one of the dominant groups, implying their potential roles in the cleanup of heavier parts of the heavy crude oil. After the increase of bacteria belonging to Alphaproteobacteria and Candidate division OD1, the Bacteroidetes bacteria affiliated to genera *Salegentibacter* and *Owenweeksia* in Flavobacteriales (T-RF of 309 bp) and *Salegentibacter* relatives (T-RF of 315 bp)

started to increase at day 70 and became dominant at day 140 (Figs. 5 and 7f). It has been reported that the genus *Flavobacterium* in the Bacteroidetes phylum was capable of degrading hydrocarbons (Salinas-Martínez et al., 2008), hence Bacteroidetes bacteria might have contributed to the continuous removal of the residual TPH in this study.

Unexpectedly, although *Marinobacter* relatives dominated in both the background soil and in the RC soil, they decreased considerably in the biostimulated soil in microcosm R1 (Fig. 7b). The species in *Marinobacter* have been reported as hydrocarbon-degrading bacteria and frequently found in marine environments contaminated with oil (Gerdes et al., 2005; Guo et al., 2007; Kasai et al., 2001). However, they were unable to tolerate high concentration of oil (Gauthier et al., 1992; Melcher et al., 2002). In the present study, heavily polluted soil (TPH of 37.85 g/kg soil), Tween 80 and bulking agent amended in microcosm R1 improved the availability of the heavy crude oil, which might in turn increased the toxicity of oil to *Marinobacter* and, as a result, a larger *Marinobacter* population was maintained in the microcosm RC with a low bioavailability of heavy crude oil.

In conclusion, the Yellow River Delta saline soil was dominated by the bacteria belonging to Gammaproteobacteria and Bacteroidetes, which showed high resistance and resilience to the pollution of the typical heavy crude oil from Shengli Oilfield. The combined stimulation treatments strongly affected the bacterial community structure, posing remarkable positive or negative impacts on most of the dominant bacteria. In addition, the combined stimulation treatments contributed to a much higher TPH removal efficiency as well as significant changes of potential hydrocarbon degrading bacteria in the following succession. The *Alcanivorax*-related bacteria, which played a key role at the first stage of crude oil biodegradation, were firstly stimulated, followed by the bacteria belonging to Alphaproteobacteria. Once the majority of the readily degradable oil components were depleted, *Alcanivorax*-related bacteria started to decrease and bacteria in Alphaproteobacteria and Candidate division OD1 gradually increased and finally dominated the oil-degraders for
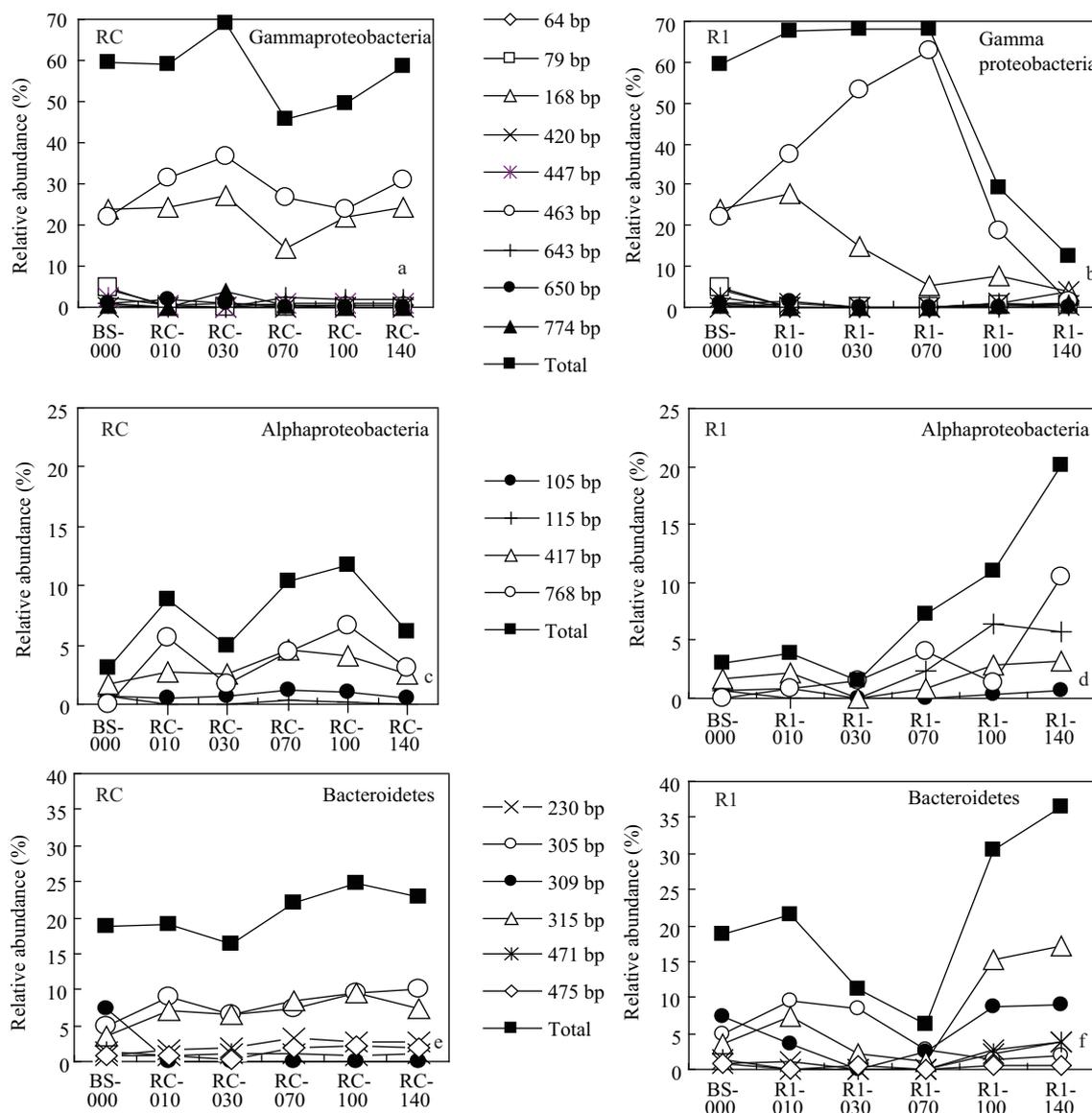
Fig. 7 Dynamics of relative abundance of the bacterial terminal restriction fragments (T-RFs) classified to Gamma- and Alphaproteobacteria and Bacteroidetes for microcosm RC and microcosm R1. The horizontal abscissa is marked by the name of the soil followed by the days of sampling as described in Fig. 5.

decomposition of the remaining constituents of the heavy crude oil. This implies different stimulation strategies could be developed according to the characteristics of bacteria so as to modulate the bacterial community for the enhanced TPH removal and improved bioremediation.

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