

1 **Identification of Benzo[a]pyrene(BaP)-Metabolizing Bacteria in Forest Soils**  
2 **Using DNA-Based Stable-Isotope Probing**

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14 **Running Title:** BaP Degraders Examined with SIP

15

16 **ABSTRACT**

17 DNA-based stable-isotope probing (DNA-SIP) was used in this study to investigate  
18 the uncultivated bacteria with benzo[a]pyrene (BaP) metabolism capacities in two  
19 Chinese forest soils (Mt. Maoer in Heilongjiang Province and Mt. Baicaowa in Hubei  
20 Province). Three different phylotypes were characterized with responsibility for BaP  
21 degradation, none of which was previously reported as BaP-degrading  
22 microorganisms by SIP. In Mt. Maoer soil microcosms, the putative BaP degraders  
23 were classified as belonging to the genus *Terrimonas* (family *Chitinophagaceae*, order  
24 *Sphingobacteriales*), whereas *Burkholderia* were the key BaP degraders in Mt.  
25 Baicaowa soils. The addition of metabolic salicylate significantly increased BaP  
26 degradation efficiency in Mt. Maoer soils, and the BaP-metabolizing bacteria shifted  
27 to the microorganisms in the family *Oxalobacteraceae* (genus unclassified).  
28 Meanwhile, salicylate addition did not change either BaP degradation or putative BaP  
29 degraders in Mt. Baicaowa. Polycyclic aromatic hydrocarbon-ring hydroxylating  
30 dioxygenase (PAH-RHD) genes were amplified, sequenced and quantified in the  
31 DNA-SIP <sup>13</sup>C heavy fraction to further confirm the BaP metabolism. By discussing  
32 the microbial diversity and salicylate additive effects on BaP degradation across  
33 different soils, the results increased our understanding of BaP natural attenuation and  
34 provided possible approach to enhance the bioremediation of BaP-contaminated soils.

35

36 **Keywords:** stable isotope probing (SIP), benzo[a]pyrene (BaP), forest soil,

37 PAH-RHD gene, salicylate

38

## 39 INTRODUCTION

40 Polycyclic aromatic hydrocarbons (PAHs), a class of persistent organic pollutants  
41 (POPs), enter the environment through both natural and anthropogenic pathways.

42 PAHs are released into the environment by means of natural processes such as forest  
43 fires and direct biosynthesis under the action of microbes and plants (1). The primary  
44 artificial source of PAHs is the incomplete combustion of organic matter at high  
45 temperatures caused by human activities (2). Their presence in the environment poses  
46 a severe threat to public and ecosystem health because of their known acute toxicity,  
47 and mutagenic, teratogenic, and carcinogenic features; they are therefore classified as  
48 priority pollutants by the U.S. Environmental Protection Agency (3). Furthermore, the  
49 persistence and genotoxicity of PAHs increase with molecular weight, and the  
50 presence of high-molecular-weight (HMW) PAHs in the environment is of greater  
51 concern. Benzo[a]pyrene (BaP), a representative HMW PAH with a five-ring structure,  
52 is a widespread pollutant with potent mutagenic and carcinogenic properties (4, 5).  
53 BaP is therefore identified as the first class of “human carcinogens” according to the  
54 report of the World Health Organization (WHO) International Agency for Research on  
55 Cancer (6). Generally, in soils with no industrial contamination, BaP concentrations  
56 vary from 3.5 to 3700 µg/kg, with a median concentration of 16 µg/kg soil; in  
57 contaminated soils and sediments, BaP concentrations range from 82 to 536 mg/kg  
58 (7).

59 Bacteria possessing the ability of BaP utilization are readily isolated from

60 contaminated soils or sediments, and most of our current knowledge about BaP  
61 metabolism by microbes has been gained from such isolates (8-10). The functional  
62 PAH-RHD genes (encoding PAH-ring hydroxylating dioxygenase enzymes) have also  
63 been examined to understand BaP degradation mechanisms, such as *nah*, *pah*, *arh* and  
64 *phn* genes in Gram-negative (GN) bacteria and the evolutionarily correlated *nid*, *nir*,  
65 *phd* and *nar* genes in Gram-positive (GP) bacteria, which are responsible for the first  
66 step of PAHs (naphthalene, phenanthrene, anthracene and pyrene) hydroxylation  
67 under aerobic conditions (11, 12). Terminal dioxygenase is the component of  
68 PAH-RHD, composed of large  $\alpha$  and small  $\beta$  subunits. The genes coding for the  
69 mononuclear iron-containing catalytic domain (a conserved regions) of PAH-RHD $\alpha$   
70 ( $\alpha$ -subunit) have been widely used for studying RHD diversity and the  
71 PAH-degradation potential by bacteria in environment (11). In addition to the research  
72 on the genes involved in PAH degradation, studies using culture-dependent tools to  
73 investigate the degrading capacity of BaP degraders showed that rare bacteria were  
74 capable of metabolizing BaP without metabolic intermediate additives (9, 10, 13); in  
75 most cases, BaP degradation is stimulated by the addition of some intermediates  
76 produced during BaP metabolism (14, 15). These intermediates possess the ability of  
77 stimulating PAH dioxygenase activity and are capable of supplying electrons for  
78 nicotinamide adenine dinucleotide (NADH) coenzymes, which are necessary for the  
79 functions of oxygenase enzymes, to initiate aerobic PAH degradation (8, 16).  
80 Salicylate is a classic intermediate, inducing PAHs metabolism of PAHs-degrading  
81 bacteria and selectively stimulating their growth (8, 16, 17). The addition of salicylate

82 to contaminated soils or sediments has been proposed as a means of encouraging PAH  
83 degradation during bioremediation (16). *Sphingomonas yanoikuyae* JAR02 was  
84 reported to completely remove BaP (1.2 mg/L) within 20 h in the aqueous phase with  
85 additive salicylate (8). Additionally, *Pseudomonas saccharophila* P15 isolated from  
86 creosote-contaminated soil improved BaP removal after the addition of salicylate,  
87 which acted as the inducer of PAH dioxygenase activity (16).

88 The traditional culture-dependent approaches, such as isolating and cultivating  
89 target bacteria in the laboratory, suffer from the fact that <1% of the soil  
90 microorganisms are cultivable. The process underestimates the diversity of the  
91 prokaryotes and fails to capture the true nature of the complex interactions within  
92 microbial communities at a specific site. The uncultured bacteria may possess an  
93 unexplored reservoir of novel and valuable gene-encoding catalysts that benefit  
94 bioremediation, industry, and medicine (18-20). Furthermore, with our limited  
95 understanding of the actual biota, cultivation presents challenges for field  
96 bioremediation (21). In recent years, stable-isotope probing (SIP) has emerged as a  
97 culture-independent method to identify microorganisms capable of utilizing specific  
98 substrates in complex environments. Microbial populations responsible for the  
99 degradation of targeted contaminants are labeled by stable isotopes and then  
100 characterized (22-25). To date, many bacteria have been successfully identified by SIP  
101 with their unique capabilities of metabolizing phenolic compounds and PAHs, such as  
102 naphthalene (25, 26), anthracene (27), phenanthrene (26), pyrene (23, 28),  
103 fluoranthene (29), benz[a]anthracene (29), biphenyl (30, 31), phenol (31), and

104 benzoate (31). However, to our knowledge, no study till now has examined  
105 BaP-degrading bacteria successfully using SIP.

106 In the present study, to investigate the microorganisms responsible for BaP  
107 degradation in the uncontaminated soil, <sup>13</sup>C-DNA-targeted SIP was applied to two  
108 Chinese forest soils from Mt. Maoer and Mt. Baicaowa. The influence of salicylate on  
109 BaP biodegradation was further studied, as well as its role in functional microbial  
110 community dynamics. By sequencing and quantifying (by real-time polymerase chain  
111 reaction, qPCR) PAH-RHD $\alpha$  genes in <sup>13</sup>C-DNA enriched fraction, our work further  
112 revealed the BaP metabolism in uncontaminated soil and the stimulating effects of  
113 salicylate addition varying among different soils. To our knowledge, this study  
114 successfully apply the culture-independent SIP technique to characterize  
115 BaP-degrading bacteria in forest soil, and provides an important contribution to the  
116 understanding of BaP biodegradation in complex communities and the bioremediation  
117 of HMW PAHs-contaminated soil.

118

## 119 MATERIALS AND METHODS

120 **Soil samples.** Soil samples were collected from Mt. Maoer (45°22'48"N,  
121 127°40'48"E) in Heilongjiang Province, and Mt. Baicaowa (40°48'36"N, 117°35'60"E)  
122 in Hebei Province, China. The pH and total organic carbon content were 7.5 and 16.0%  
123 (Mt. Maoer), and 6.0 and 12.3% (Mt. Baicaowa), respectively. Before use, large  
124 objects in the soils, such as stones and debris, were removed manually in the  
125 laboratory. The soil samples were then homogenized, sieved through a 2-mm

126 pore-size screen, and prepared for the BaP-degradation treatment.

127 **Setup of BaP-degrading microcosms.** Both non-salicylate and salicylate-additive  
128 treatments were included for the two forest soils. Hereafter, BS and B represent the  
129 Mt. Baicaowa soils amended with and without salicylate, and MS and M denote the  
130 Mt. Maoer soils amended with and without salicylate, respectively. The experiments  
131 were conducted as follows: Three grams of soil (dry weight) was placed in a 150-mL  
132 serum bottle containing 10 mL phosphate-buffered mineral medium (32). The bottles  
133 were sealed with rubber stoppers and compressed with an aluminum seal. The  
134 unlabeled BaP (99%) or  $^{13}\text{C}$ -labeled BaP ( $^{13}\text{C}_4$ -BaP, 99%, Fig. S1 shows the position  
135 of  $^{13}\text{C}$ -labeled carbons), both from Cambridge Isotope Laboratories, Inc. and  
136 dissolved in nonane with a final concentration of 100 mg/l, was added to the  
137 respective bottles using a gastight syringe to give a final BaP concentration of 1  
138 mg/kg. For salicylate-additive treatments, the final salicylate concentration was 10  
139 mg/kg. Two negative control treatments were included as no-carbon-source (CK) and  
140 non-bioactive (sterilized by  $\gamma$ -irradiation,  $^{12}\text{C}$ -NB). Two positive treatments were  
141 amended with  $^{12}\text{C}$ - and  $^{13}\text{C}$ -labeled BaP carbon sources, named as  $^{12}\text{C}$ -BT and  $^{13}\text{C}$ -BT,  
142 respectively. Eight samples were prepared for each treatment. The microcosms were  
143 incubated at room temperature ( $\sim 25\text{ }^\circ\text{C}$ ) with reciprocal shaking at a speed of 120  
144 rpm/min. On Day 7, 14, 28, and 42 after incubation, two samples from each treatment  
145 were sacrificed for BaP analysis and DNA extraction, respectively. All stock solutions  
146 were filtered through 0.2- $\mu\text{m}$  pore-size filters and stored in dark-brown containers. To  
147 prepare sterile controls, soils were  $\gamma$ -irradiated (50 kGy) for 2 h before use.

148       **BaP analysis.** The <sup>12</sup>C-NB, <sup>12</sup>C-BT and <sup>13</sup>C-BT samples were prepared for BaP  
149 analysis using the following steps: The serum bottles were frozen at -20 °C overnight,  
150 followed by freeze-drying using a vacuum freeze dryer. The dry soil samples were  
151 then homogenized, pulverized, spiked with 1,000 ng of deuterated PAHs as surrogate  
152 standards and extracted with dichloromethane (DCM) in a Soxhlet apparatus for 48 h,  
153 with the addition of activated copper to remove sulfur. The extract was concentrated  
154 to ~0.5 mL after solvent exchange to hexane. The soil extracts were purified in a  
155 multilayer silica gel/alumina column (8 mm i.d.) filled with anhydrous Na<sub>2</sub>SO<sub>4</sub> (1 cm),  
156 neutral silica gel (3 cm, 3% w/w; deactivated), and neutral alumina (3 cm, 3% w/w;  
157 deactivated; from top to bottom) via elution with 15 mL hexane/DCM (1:1, v/v). After  
158 concentrating to ~50 µL under a gentle stream of N<sub>2</sub>, 1,000 ng of hexamethylbenzene  
159 was added as an internal standard prior to analysis.

160       BaP was detected on an Agilent 7890 gas chromatograph equipped with a  
161 capillary column (DB-5MS, 30 m, 0.25 mm, 0.25 µm) and a mass spectrometric  
162 detector (MSD, Agilent 5975). One microliter of sample was injected in splitless  
163 mode with a 10-min solvent delay time. High-purity helium was used as the carrier  
164 gas at a flow rate of 1.83 mL/min. The temperature of the injector and transfer lines  
165 was 290 and 300 °C, respectively. The initial oven temperature was set at 60 °C for 1  
166 min, rising to 290 °C at a rate of 3 °C/min, and was subsequently held constant for 20  
167 min. PAH standards were used to quantify BaP. Instrumental performance was  
168 subjected to quality-control calibration with the standards after each set of eight  
169 samples had been analyzed. Six PAHs standard concentrations were used to derive the



170 calibration curves. Concentrations were corrected using reference to surrogate  
171 recovery levels.

172 **DNA extraction and ultracentrifugation.** Samples from <sup>12</sup>C-BT and <sup>13</sup>C-BT  
173 were both prepared for biotic analysis at 7, 14, 28, and 42 days of cultivation. The  
174 total genomic DNA was extracted from 1.0 g soils with triplicates using the  
175 Powersoil® DNA Isolation Kit (MO BIO Laboratories, Inc.) according to the  
176 manufacturer's protocol. DNA concentrations were determined using an ND-2000  
177 UV-Vis spectrophotometer (NanoDrop Technologies). Subsequently, ~10,000 ng  
178 DNA was added to Quick-Seal polyallomer tubes (13 × 51 mm, 5.1 mL, Beckman  
179 Coulter), along with a Tris-EDTA (TE, pH 8.0)/cesium chloride (CsCl) solution.  
180 Before the tubes were sealed with cordless Quick-Seal® Tube Topper (Beckman  
181 Coulter), the average buoyant density (BD) of all prepared gradients was determined  
182 with an AR200 digital refractometer (Leica Microsystems Inc.) and adjusted to ~1.77  
183 g/mL by adding a CsCl solution or Tris-EDTA buffer, if necessary. The tubes were  
184 transferred to an ultracentrifuge (Optima L-100XP, Beckman Coulter) and centrifuged  
185 at 178,000 × g (20 °C) for 48 h. Following centrifugation, 150-μL fractions were  
186 collected from each tube using a fraction recovery system (Beckman Coulter). The  
187 BD of each fraction was measured, and the CsCl was further removed by  
188 glycogen-assisted ethanol precipitation (33).

189 **PCR and terminal restriction fragment length polymorphism (TRFLP).** The  
190 fractions were subjected to terminal restriction fragment length polymorphism  
191 (TRFLP) analysis using standard procedures (24). Briefly, DNA was amplified with

192 27F-FAM (5'-AGAGTTTGATCMTGGCTCAG; 5' end-labeled with  
193 carboxyfluorescein) and 1492R (5'-GGTTACCTTGTTACGACTT) using the  
194 following PCR program: initial melting at 94 °C for 5 min, 30 amplification cycles at  
195 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1.5 min, with a final extension at 72 °C for  
196 10 min. After amplification, the presence of PCR products was confirmed by 1%  
197 agarose gel electrophoresis. The PCR products were purified using an EZNA  
198 Cycle-Pure Kit (Omega Bio-Tek, Inc.) following the manufacturer's instructions and  
199 digested with *Hae*III (New England Biolabs) for 4–5 h at 37 °C. One nanogram of  
200 each labeled PCR product was analyzed on an ABI 3730 Genetic Analyzer (Applied  
201 Biosystems) running Peak Scanner software version 1.0 (Applied Biosystems). A  
202 GeneScan™ ROX 500™ set of internal standards (Applied Biosystems) was used.  
203 The percent abundance of each fragment was determined as previously described  
204 (30).

205 **Identification of the PAH-RHD<sub>α</sub> gene in microcosms and enumeration in SIP**  
206 **fractions.** As the PAH-RHD<sub>α</sub> genes possessed by GN and GP bacteria do not belong  
207 to a monophyletic cluster (12), the presence of the PAH-RHD<sub>α</sub> genes was  
208 investigated using PAH-RHD<sub>α</sub> GP primers (641F  
209 5'-CGGCGCCGACAAYTTYGTNGG, 933R  
210 5'-GGGGAACACGGTGCCRTGDATRAA) and PAH-RHD<sub>α</sub> GN primers (610F  
211 5'-GAGATGCATACCACGTKGGTTGGA, 911R 5'-  
212 AGCTGTTGTTCGGGAAGAYWGTGCMGTT) (32) on the heavy DNA fractions  
213 from B, BS, M and MS microcosms, respectively. A gradient PCR was performed

214 with annealing temperatures ranging from 52 to 62 °C. Amplification reactions were  
215 carried out in a volume of 50 µL, as previously described (12).

216 The copy number of the PAH-RHD $\alpha$  sequences in fractions from <sup>13</sup>C-labeled and  
217 unlabeled DNA was determined by qPCR using the PAH-RHD $\alpha$  GP primers, because  
218 only one strong and specific amplicon amplified with PAH-RHD $\alpha$  GP primers was  
219 produced from the heavy fractions in M and MS microcosms in this work. The PCR  
220 mix contained 10 µL SYBR Green® PCR Premix Ex Taq™ II (TaKaRa Bio) and 1 µL  
221 DNA template in a final volume of 20 µL. A standard curve was obtained by  
222 producing 10 dilution series of plasmid pGEM-T Easy Vector sequences (10<sup>2</sup>–10<sup>8</sup>  
223 copies; Promega) containing the PAH-RHD $\alpha$  gene detected in M and MS treatments  
224 with the PAH-RHD $\alpha$  GP primers (641F 5'-CGGCGCCGACAAYTTYGTNGG, 933R  
225 5'-GGGGAACACGGTGCCRTGDATRAA). The amplification reactions were  
226 conducted using a two-step method in a 48-well optical plate on an Eco™ real-time  
227 PCR system (Illumina) as follows: denaturation for 10 min at 95 °C, followed by 40  
228 cycles of 10 s at 95 °C, and 30 s at 60 °C; then the SYBR Green signal intensities were  
229 measured after the 30 s step at 60 °C. At the end of the real-time PCR, a melting curve  
230 analysis was performed by increasing the temperature from 55 to 95 °C. For each  
231 DNA sample, the average of three replicates was determined as the copy number per  
232 fraction.

233 **Sequencing of partial PAH-RHD $\alpha$  and 16S rRNA genes.** The 16S rRNA genes  
234 from the heavy fractions (<sup>13</sup>C-labeled DNA, marked with ☆ in Fig. 1)  
235 corresponding to the peak in Fig. 1A- Fig. 1D with the BD value of 1.7198 g/ml,

236 1.7203 g/ml, 1.7165 g/ml and 1.7100 g/ml, as well as the amplicons generated with  
237 the PAH-RHD $\alpha$  GP primer pair in M and MS treatments, were cloned and sequenced.  
238 Briefly, the 16S rRNA gene was amplified using a similar process to that described  
239 above, except the 27F-FAM primer was replaced by 27F. The purified PCR products  
240 were cloned into a pGEM-T Easy Vector and transformed into *Escherichia coli* JM  
241 109 (TaKaRa Bio). *E. coli* clones were then grown on Luria-Bertani medium  
242 solidified with 15 g/L agar in the presence of 50  $\mu$ g/L ampicillin for 16 h at 37°C, and  
243 finally 100 clones with inserts were selected to be sequenced. The plasmids with  
244 target genes were extracted using an EZNA Plasmid Mini-Kit (Omega Bio-Tek, Inc.),  
245 and the recombinants were selected using 0.8% agarose gel electrophoresis and  
246 sequenced on an ABI 3730 genetic analyzer using M13 primers. Sequence similarity  
247 searches and alignments were performed using the Basic Local Alignment Search  
248 Tool (BLAST) algorithm (National Center for Biotechnology Information) and  
249 Molecular Evolutionary Genetics Analysis (MEGA 5.1).

250 The obtained 16S rRNA gene and the partial RHD gene sequences are available in  
251 GenBank (accession numbers KM267480–KM267482 for partial 16S rRNA gene  
252 sequences of a 196-bp terminal restriction fragment (TRF) in B and BS treatments,  
253 450-bp TRF in M treatment, and 216-bp TRF in MS treatment; KM267486 was used  
254 for the partial PAH-RHD $\alpha$  gene sequence).

255

## 256 **RESULTS**

257 **BaP biodegradation in soils.** BaP biodegradation in the four treatments (B, BS,

258 M and MS) is briefly listed in [Table 1](#). The recovery rates of BaP during the extraction  
259 procedure were 80%-90% in this work ([Table S2](#)). The BaP concentration in sterile  
260 treatments showed less decline comparing to unsterilized treatments. For instance, the  
261 residual BaP were 80.6%, 72.6%, 81.0% and 69.7% for the <sup>12</sup>C-NB of B, BS, M and  
262 MS microcosms, respectively. Significant BaP biodegradation was observed in the  
263 unsterilized microcosms. For B and BS treatments ([Table 1](#)), BaP degradation  
264 achieved 16.6% and 18.1% respectively after 7 days cultivation, and 33.4% and 31.1%  
265 of BaP was removed after 28 days. No significant BaP degradation difference was  
266 found between the B and BS treatments throughout the whole process, indicating the  
267 limited impacts of salicylate on the capacities of BaP-degrading bacteria. The BaP  
268 degradation in M and MS treatments was much faster than those from Mt. Baicaowa.  
269 For example, 32.1% and 47.4% of BaP was removed in M and MS samples after 14  
270 days cultivation, compared to the corresponding degradation efficiency of 24.9% and  
271 23.0% in B and BS samples. Salicylate significantly accelerated BaP biodegradation  
272 in soils from Mt. Maoer, where BaP removal efficiency was 32.1%, 43.7%, and 45.7%  
273 in M treatments at 14, 28, and 42 days, compared to the corresponding 47.4%, 52.5%,  
274 and 55.8% in MS treatments ([Table 1](#)).

275 **Microbial structure analysis via SIP and TRFLP.** DNA extracts from <sup>12</sup>C-BT  
276 and <sup>13</sup>C-BT soil samples were subjected to ultracentrifugation and fractionation,  
277 followed by TRFLP for each fraction. The organisms responsible for <sup>13</sup>C assimilation  
278 were detected by the relative abundances of specific TRFs between the control  
279 (<sup>12</sup>C-BT) and the treatment with <sup>13</sup>C-labeled BaP (<sup>13</sup>C-BT) at all three sampling points

280 for each fraction.

281 In B microcosms, the TRFLP results (Fig. 1) indicated that the 196-bp *HaeIII* TRF  
282 at higher buoyant densities (1.7176–1.7328 g/mL) was enriched at 7, 14, and 28 days  
283 (16.6%, 24.9%, and 33.4% BaP removal, respectively) in <sup>13</sup>C-BT samples and that its  
284 relative abundance increased with time. Such enrichment and increasing trend were  
285 not observed in the <sup>12</sup>C-BT controls. Additionally, the enrichment of the 196-bp  
286 *HaeIII* TRF in the heavy fractions was also supported by their higher fluorescence  
287 intensity in the 28-day treatments (Fig. S2A), suggesting that <sup>13</sup>C was incorporated by  
288 the microorganisms represented by the 196-bp *HaeIII* TRF. To identify the  
289 BaP-degrading bacteria and obtain the phylogenetic affiliation of the 196-bp *HaeIII*  
290 TRF, the 16S rRNA clone library derived from the <sup>13</sup>C heavy fractions was sequenced,  
291 and the clones with the 193-bp *HaeIII* TRF cut site matched the TRFLP results for the  
292 196-bp TRFs. The slight difference (2–3 bases) between the measured fragment  
293 lengths and those predicted using sequence data has been noted in previous studies  
294 (24, 34, 35). Based on the comparative analyses of 16S rRNA, the bacteria  
295 represented by the 196-bp TRF were classified as members of the genus *Burkholderia*  
296 (Fig. S3). Additionally, a partial sequence with the predicted 213-bp *HaeIII* cut site in  
297 the clone library was related to the class *Acidobacteria*. An additional member with  
298 the predicted 206-bp *HaeIII* cut site in the clone library was related to the genus  
299 *Rhodanobacter* (Table S1).

300 In BS microcosms with salicylate addition, the bacteria represented by the 196-bp  
301 *HaeIII* TRF were involved in BaP biodegradation, as shown by DNA-SIP. Fig. 1A

302 illustrates a clear increasing relative abundance of 196-bp *HaeIII* TRF at higher BD  
303 (>1.7203 g/mL) in the <sup>13</sup>C-BT samples compared to the <sup>12</sup>C-BT at 7, 14, and 28 days.  
304 Furthermore, in the <sup>13</sup>C-BT samples after 28 days cultivation, the BD value of the  
305 strongest fluorescence intensity of the 196-bp *HaeIII* TRF was higher than that of the  
306 <sup>12</sup>C-BT samples (Fig. S2B). When the 16S rRNA clone library derived from the <sup>13</sup>C  
307 heavy fractions was inspected (Table S1), 43 of the 100 clones with the predicted  
308 193-bp *HaeIII* cut site fell in the genus *Burkholderiales*. These clones were associated  
309 with BaP degradation, the same as the BaP degraders in B treatment. Additionally,  
310 three different clones with the predicted 362-, 217-, and 202-bp *HaeIII* cut sites were  
311 classified within the order *Burkholderiales*, and one clone with the predicted 212-bp  
312 *HaeIII* cut site shared 100% similarity with strain *Acidobacteria* Gp3, which also  
313 occurred in the heavy fraction of B treatment.

314 In M microcosms, the TRFLP fraction profiles (Fig. 1C) and the fluorescence  
315 intensity at 42 days (Fig. S2C) indicated relatively more abundant 450-bp *HaeIII* TRF  
316 in the heavy fractions (BD >1.7121 g/mL) of <sup>13</sup>C-BT samples, but not in <sup>12</sup>C-BT  
317 samples. Furthermore, the relative abundance of the 450-bp *HaeIII* TRF in the heavy  
318 fraction increased with time in <sup>13</sup>C-BT samples, and the magnitude of the increase  
319 was largest for the sample that had been left the longest. For the <sup>12</sup>C-BT samples, the  
320 increase occurred in the light fraction (BD <1.7045 g/mL), indicating <sup>13</sup>C-BaP  
321 assimilation by the bacteria represented by the 450-bp *HaeIII* TRF. However, this was  
322 not the dominant TRFLP fragment in the heavy fractions from <sup>13</sup>C-BT samples, and  
323 the other three TRFs (237, 372, and 215 bp) were the major members. Nevertheless,

324 those microorganisms were not responsible for  $^{13}\text{C}$ -BaP degradation since a similar  
325 abundance was found in the heavy fractions of the  $^{12}\text{C}$ -BT samples. The sequence of  
326 16S rRNA clone libraries with the predicted 447-bp cut site fit well with the TRFLP  
327 results. They were assigned to the genus *Terrimonas* (phylum *Bacteroidetes*, class  
328 *Sphingobacteria*, order *Sphingobacteriales*, family *Chitinophagaceae*), belonging  
329 most closely to the *Flavisolibacter ginsengiterrae* strain Gsoil 492 (Fig. S4). Three  
330 clones with predicted 237-, 372-, and 215-bp *Hae*III cut sites also appeared in the 16S  
331 rRNA clone library derived from the  $^{13}\text{C}$  heavy fractions (Table S1), and they were  
332 classified in the genus *Spartobacteria incertae sedis*, class *Acidobacteriaceae* and  
333 family *Oxalobacteraceae*, respectively.

334 In MS treatment with salicylate addition, the 216-bp *Hae*III TRF was involved in  
335 the BaP biodegradation, and was enriched as the dominant TRF in the heavy fractions  
336 (BD >1.7056 g/mL) at 28 and 42 days (Fig. 1D and Fig. S2D). An increasing relative  
337 abundance was also observed for 14–42 days of cultivation in treatments amended  
338 with  $^{13}\text{C}$ -BaP, but not in  $^{12}\text{C}$ -BT samples (Fig. 1D). Such increase suggested that  
339 microorganisms represented by the 216-bp TRF were responsible for  $^{13}\text{C}$  substrate  
340 uptake ( $^{13}\text{C}$ -BaP degradation). Additionally, the relative abundance and fluorescence  
341 intensity of the 77-, 200-, and 450-bp TRFs (Fig. S2D) were also high in the heavy  
342 fractions, but lower than the 216-bp TRF in the  $^{13}\text{C}$ -BT samples. The BD values of the  
343 three TRFs and their trends of relative abundance were similar between  $^{13}\text{C}$ -BT and  
344  $^{12}\text{C}$ -BT treatments at the three sampling times. Hence, the microorganisms  
345 represented by the three TRFs were not directly involved in BaP degradation, and the



346 large proportion of TRFs in the fractions might be due to their tolerance to BaP.  
347 Clones with the 216-bp *Hae*III cut site from 16S rRNA clone libraries matched the  
348 TRFLP results, classified as members of the family *Oxalobacteraceae* (phylum  
349 *Proteobacteria*, class *Betaproteobacteria*, order *Burkholderiales*) , also present in  
350 MS <sup>13</sup>C-BaP treatment, and sharing 98% sequence similarity with *Janthinobacterium*  
351 *lividum* strain DSM 1522 (Fig. S5). Clones with 77-, 200-, and 447-bp *Hae*III cut sites  
352 were classified in the order *Actinomycetales*, family *Burkholderiaceae*, and genus  
353 *Terrimonas*, respectively (Table S1-MS).

354 **Occurrence and quantification of PAH-RHD $\alpha$  genes in the SIP fractions.** In B,  
355 BS, M, and MS treatments, PAH-RHD $\alpha$  GP amplicons were only detected in the  
356 heavy fractions from the <sup>13</sup>C-BaP-amended M and MS microcosms with the primer  
357 pair of 641f and 933r (Fig. 2), although both PAH-RHD $\alpha$  GP and PAH-RHD $\alpha$  GN  
358 primers were used to amplify PAH-RHD $\alpha$  genes in all treatments. In both treatments,  
359 the PAH-RHD $\alpha$  gene (PAH-RHD $\alpha$ -M) sequences shared 99% similarity with those of  
360 an uncultured strain (KF656719.1), and also high sequence similarity with the  
361 affiliation to the genus *Mycobacterium* which was capable of degrading BaP (15, 36).  
362 Recently, the microbial metabolism of low-molecular-weight (LMW) PAHs with no  
363 more than three rings has been studied extensively, including their metabolic  
364 pathways, and enzymatic and genetic regulation (7). However, little is known about  
365 the metabolic pathways and genes related to BaP degradation and other HMW-PAHs  
366 (37). Therefore, not all the functional genes derived from the active BaP-degrading  
367 bacteria could be detected by the primer sets used in this study.

368 PAH-RHD $\alpha$ -M genes in M and MS treatments were quantified against each  
369 density-resolved fraction (Fig. 3). A marked enrichment of PAH-RHD $\alpha$ -M genes in  
370 the heavy fractions (BD >1.7200 g/mL) was observed in the <sup>13</sup>C-BaP-amended soils  
371 in MS treatment, indicating that the BD value of PAH-RHD $\alpha$ -M increased with  
372 <sup>13</sup>C-BaP degradation efficiency. In <sup>12</sup>C-BaP control, the majority of the  
373 PAH-RHD $\alpha$ -M genes were found in fractions with BD <1.7200 g/mL (Fig. 3A). For  
374 M treatment, no significant difference was observed between the <sup>13</sup>C-BT and <sup>12</sup>C-BT  
375 samples due to the limited changes in the PAH-RHD $\alpha$ -M genes and BD value (Fig.  
376 3B). Hence, the detected PAH-RHD $\alpha$ -M genes were associated with BaP degradation  
377 in MS but not in M treatment, attributing to the salicylate addition, which  
378 significantly promoted the expression of PAH-RHD $\alpha$ -M genes, stimulated  
379 PAH-RHD $\alpha$ -M encoding bacteria and improved BaP biodegradation.

380

## 381 DISCUSSION

382 **Microorganisms responsible for the BaP degradation.** DNA-SIP has been  
383 widely applied to the identification of pollutant degraders in numerous environmental  
384 media and with an ever-expanding pool of compounds (22, 27, 38). In the present  
385 study, the coupling of DNA-SIP and TRFLP techniques revealed the bacteria  
386 correlated with BaP metabolism in soils from Mt. Maoer and Mt. Baicaowa. Bacteria  
387 represented by 196-bp TRF were classified as members of the genus *Burkholderia*,  
388 and involved in BaP degradation in both B and BS treatments. *Burkholderia*-related  
389 bacteria have been linked with PAHs (39) and biphenyl (40) biodegradation in soil.

390 As the dominant genus with key roles in the degradation of oil components (41),  
391 *Burkholderia* was found to be capable of degrading anthracene, phenanthrene,  
392 chrysene, and pyrene (7). Juhasz et al. found that a *Burkholderia cepacia* strain  
393 isolated from soil near a manufacturing gas plant could degrade BaP with pyrene as  
394 the carbon source, although only 1.4–6.2% BaP was removed after 56 days (42). A  
395 *Delftia* strain was isolated from the microbial consortium of a crude oil-contaminated  
396 soil and removed 56.6% BaP in PAH-contaminated soil after 14 days (43). To our  
397 knowledge, prior to this study, BaP degradation by *Burkholderia*-related  
398 microorganisms using DNA-SIP has not been documented.

399 In M treatment, microorganisms represented by the 450-bp TRF were correlated  
400 with BaP degradation and assigned to the genus *Terrimonas*. Sequence analysis  
401 suggested their close relationship to the *F. ginsengiterrae* strain Gsoil 492 (Fig. S4).  
402 This strain was first isolated by Yoon and Im from soil used for ginseng planting, and  
403 it has the ability for growth with 3-hydroxybenzoic or 4-hydroxybenzoic acid as the  
404 sole carbon source (44). Strains of the genus *Sphingomonas* from the family  
405 *Sphingomonadaceae*, order *Sphingomonadales*, were able to degrade BaP with  
406 different co-metabolic substances, and aqueous 1.2 mg/L BaP was completely  
407 removed within 20 h when *S. yanoikuyae* JAR02 grew on salicylate (8). Ye et al. also  
408 showed that 5% BaP was removed by *Sphingomonas paucimobilis* with fluoranthene  
409 as the co-metabolic source of carbon and energy after 168 h when the initial  
410 concentration of BaP was 10 mg/L (45). Until now, some strains affiliated to  
411 *Chitinophagaceae* were found in various environments and possessed the functions of

412 metabolizing complex organic compounds. Though the crucial roles of these strains in  
413 carbon circulation were reported (46), little was known about their PAH-degrading  
414 capabilities. The present study show that the genus *Terrimonas* correlated with BaP  
415 degradation, which expand our knowledge of this genus.

416 The bacteria involved in BaP degradation in MS microcosm amended with BaP  
417 and salicylate were affiliated most closely with the family *Oxalobacteraceae* (phylum  
418 *Proteobacteria*, class *Betaproteobacteria*, order *Burkholderiales*, 216 bp). To date,  
419 many *Pseudomonas* strains in  $\gamma$ -*Proteobacteria* have been shown to possess the  
420 capability of degrading aromatic hydrocarbons such as pyrene, phenanthrene,  
421 naphthalene, toluene, and phenol in crude oil-contaminated soil (47).  
422 *Burkholderiales*-related bacteria in  $\beta$ -*Proteobacteria* were also related to PAHs  
423 removal, and the family *Oxalobacteraceae* was widely found in PCBs- and  
424 PAHs-contaminated soils (40, 48). Huang reported that a cultivated bacterial strain  
425 from the class *Burkholderiales* could degrade PAHs (41). However, no previous  
426 studies using SIP have demonstrated the BaP-degradation capacity of  
427 *Burkholderiales*-related bacteria, and our results in Mt. Baicaowa soils suggest such  
428 possibilities.

429 The coupling of TRFLP with SIP enabled us to compare the TRFLP profiles over  
430 a range of BDs for both  $^{13}\text{C}$ -labeled and unlabeled samples at different time intervals  
431 to avoid false-positive results. For example, in the M treatment, although three clones  
432 with predicted 237-, 372-, and 215-bp *HaeIII* cut sites were dominant in the 16S  
433 rRNA clone library, and the corresponding TRFs constituted a high proportion in the

434 <sup>13</sup>C heavy fractions from <sup>13</sup>C-BaP-amended treatment, no significant difference was  
435 observed in these TRFs between the <sup>13</sup>C-BaP and <sup>12</sup>C-BaP treatments, suggesting their  
436 limited roles in *in situ* BaP degradation in our study.

437 The conflict between Gram-positive PAH-RHDa genes and detected  
438 Gram-negative BaP degraders is a puzzle. Such evolutionary distant has been  
439 explained previously as horizontal gene transfers of PAH-RHDa genes and other  
440 genes between Gram-positive and Gram-negative bacteria (49-52). For instance, the  
441 classical *nah*-like genes were shared among gram-negative bacteria (49). *aphA-3*, an  
442 antibiotic resistance gene in *Campylobacter* encoding 3'-aminoglycoside  
443 phosphotransferases modifying the structure of kanamycin, was transferred between  
444 gram-positive and gram-negative bacteria (53). In our study, SIP results could not  
445 directly affiliate the Gram-positive PAH-RHDa genes to the functional  
446 Gram-negative host, therefore not able to prove the evidence of horizontal gene  
447 transfer. Further work is suggested on the single-cell isolation and genome  
448 amplification of individual BaP degraders (54) and deeper investigation on the  
449 PAH-RHDa genes within the targeting functional species.

450 **Effects of salicylate on BaP degradation and community structure.** The  
451 addition of salicylate significantly changed the functional microbial community  
452 structure in the heavy fractions derived from the Mt. Baicaowa soils, but not the BaP  
453 degradation. The dominant species shifted from *Rhodanobacter*-related bacteria in B  
454 microcosm to *Burkholderiales*-related bacteria in BS treatment, although  
455 *Acidobacteria*-related microorganisms existed in both treatments. The bacteria

456 capable of degrading BaP (*Burkholderiales*) in B and BS treatments were detected as  
457 identical using DNA-SIP (Table S1). Nevertheless, the similar BaP removal efficiency  
458 between B and BS treatments hinted that BaP degradation was not stimulated by the  
459 functional microbial structure change and increasing *Burkholderiales*-related bacteria,  
460 consistent with a previous study by Powell (12). Since small pH variation might  
461 significantly affect the biodegradation of xenobiotics and other organic compounds in  
462 oligotrophic environments (55), the possible reason was the decreased PAHs  
463 degradation activity of dominant *Burkholderiales*-related bacteria under low pH  
464 conditions (56), like pH=6.0 in Mt. Baicaowa soils.

465 However, the TRFLP in M and MS treatments suggested that the addition of  
466 salicylate changed both functional microbial community structure in heavy fractions  
467 and BaP degradation rate (Table S1). The dominant species correlated with BaP  
468 degradation shifted from *Terrimonas* to *Oxalobacteraceae* after the salicylate addition.  
469 The functional PAH-RHD genes might change (Fig. 3), and salicylate also accelerated  
470 the BaP degradation in Mt. Maoer microcosms (Table 1). Previous studies showed a  
471 significant increase in the rate of naphthalene mineralization in soil after enrichment  
472 with salicylate spiking (38). The presence of phenanthrene and salicylate also greatly  
473 enhanced the initial removal rates of benz[a]anthracene, chrysene, and  
474 benzo[a]pyrene by *P. saccharophila* P15 (16). The addition of salicylate to  
475 PAHs-contaminated soils was shown to increase the quantity of  
476 naphthalene-degrading bacteria (57, 58), and stimulate the degradation of  
477 benzo[a]anthracene, chrysene (16), fluoranthene (16, 17), and BaP (8, 16).

478 Salicylate was also reported to sustain populations of biological control bacteria with  
479 naphthalene-degrading genes in agricultural systems (59), and various salicylate  
480 additives (spiked or slow/continuous addition) have been used to select different  
481 microbial communities (38).

482 The effects of salicylate on PAHs removal and functional microbial community  
483 structure depend on the soil properties and the bacterial profiles in the soils. Although  
484 salicylate is the central metabolite of many PAHs-degradation processes, it is not  
485 associated with some pathways, and its stimulating effect, therefore, might not be  
486 suitable for all cases of PAHs degradation. For example, the addition of salicylate had  
487 no effect on phenanthrene or pyrene removal in PAHs-contaminated soils (60). After  
488 enrichment with salicylate, the initial naphthalene mineralization rate rather than  
489 phenanthrene and BaP, was enhanced by the microbial community in a bioreactor for  
490 a PAHs-contaminated soil treatment (38). While, in uncontaminated soils, salicylate  
491 only improved pyrene removal but did not affect BaP (60), which may explain why  
492 salicylate stimulated BaP removal in Mt. Maoer soils, but not Mt. Baicaowa.

493 In summary, three phylotypes in two different forest soils were linked with BaP  
494 degradation using the culture-independent SIP technique. The addition of salicylate  
495 affected the bacteria correlated with BaP metabolism and the BaP degradation  
496 efficiency differently in the two forest soils. Besides, a new PAH-RHD $\alpha$  gene  
497 involved in BaP metabolism was detected in the salicylate-amended soils from Mt.  
498 Maoer. Our results provide a deeper understanding of the contribution of SIP to  
499 identifying the functions of uncultured microorganisms, expand our knowledge on

500 bacteria possessing the ability of BaP mineralization, and reveal specific effects of  
501 salicylate on the BaP-biodegradation process.

502

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667 **FIGURE AND TABLE LEGENDS**

668 **Table**

669 **Table 1.** Percentage of BaP remaining in soils over time

670 **Figures**

671 **Fig. 1** Relative abundance of TRFLP fragments (digested by HaeIII) assigned to *Burkholderia*  
672 (A), *Burkholderia2* (B), *Terrimonas* (C), *Oxalobacteraceae* (D) against the buoyant density  
673 gradients in B, BS, M and MS treatments.

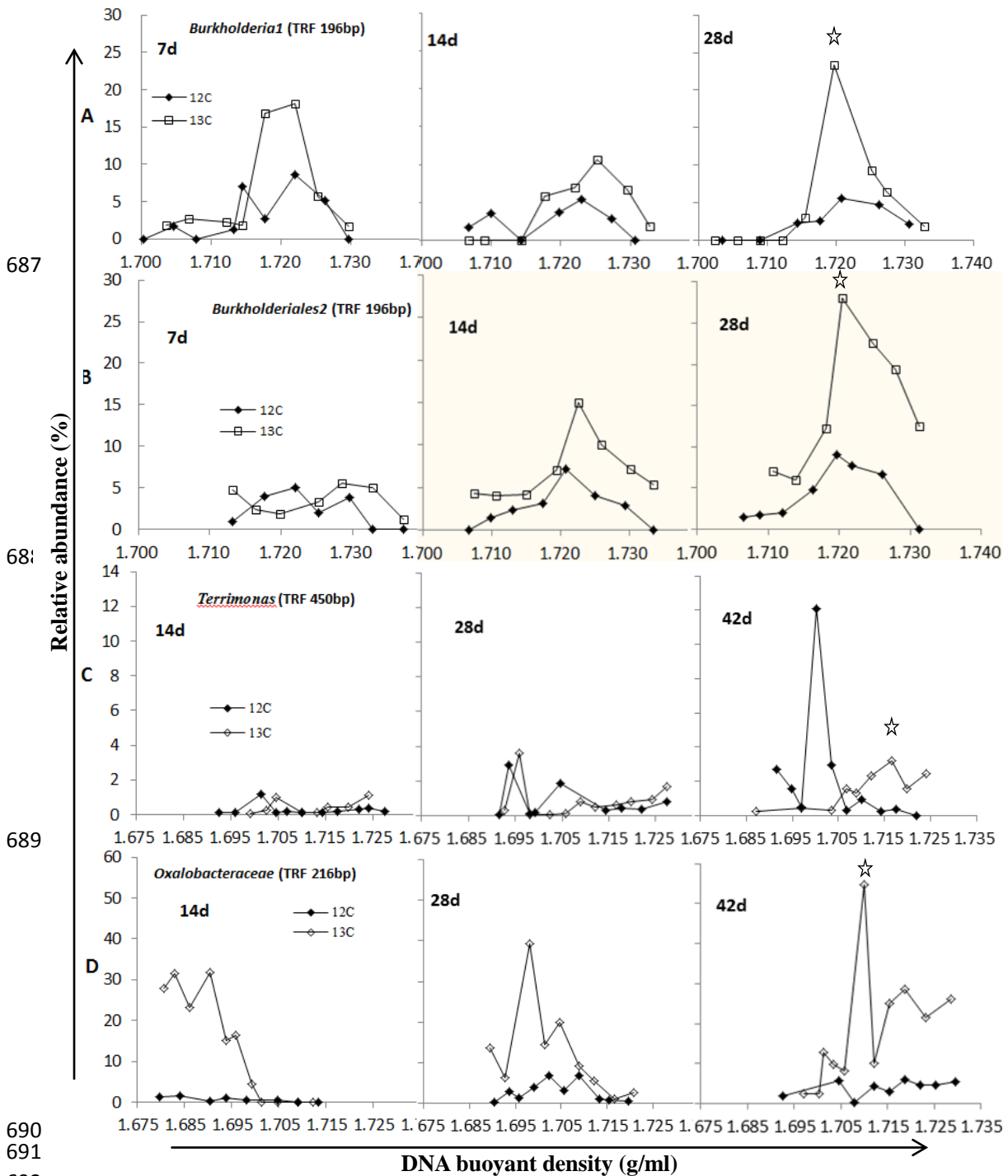
674 **Fig. 2** Phylogenetic tree of PAH-RHD $\alpha$ -M genes from MS (microcosms from Mt. Maoer soil  
675 amended with salicylate) and M (microcosms from Mt. Maoer soil without salicylate) treatments  
676 along with the closest matches in GenBank, constructed with MEGA 5.0 software using the  
677 neighbor-joining method.

678 **Fig. 3** PAH-RHD $\alpha$ -M gene copies in ultracentrifugation fractions from  $^{13}\text{C}$ -BaP and  $^{12}\text{C}$ -BaP  
679 amended microcosms determined by qPCR. A and B represent the microcosms from Mt. Maoer  
680 soil with/without salicylate addition. Figure symbols:  $\square$   $^{13}\text{C}$ -BaP (~52% BaP degraded);  $\blacksquare$   
681  $^{12}\text{C}$ -BaP (~42% BaP degraded).

**Table 1.** Percentage of BaP remaining in soils over time

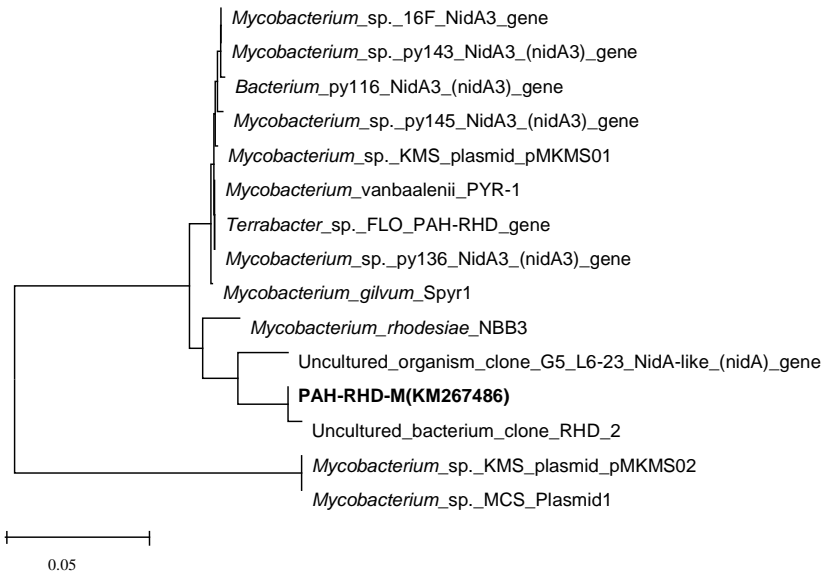
Time (days)	B(%)			BS(%)			M(%)			MS(%)		
	<sup>12</sup> C-NB	<sup>12</sup> C-BT	<sup>13</sup> C-BT	<sup>12</sup> C-NB	<sup>12</sup> C-BT	<sup>13</sup> C-BT	<sup>12</sup> C-NB	<sup>12</sup> C-BT	<sup>13</sup> C-BT	<sup>12</sup> C-NB	<sup>12</sup> C-BT	<sup>13</sup> C-BT
7	87.1	70.5	71.3	85.3	67.2	66.3	-	-	-	-	-	-
14	78.4	53.5	52.7	83.1	60.1	62.1	88.9	56.8	59.7	72.9	25.6	24.8
28	80.6	47.2	48.6	72.6	41.5	41.5	82.0	38.3	37.9	70.4	17.9	18.2
42	-	-	-	-	-	-	81.0	35.4	36.4	69.7	13.9	11.7

683 Note: <sup>12</sup>C-NB represents the non-bioactive autoclaved treatment; <sup>12</sup>C-BT represents the treatment with <sup>12</sup>C-BaP as the sole carbon sources; <sup>13</sup>C-BT represents the treatment  
684 with <sup>13</sup>C-BaP as the sole carbon sources. B and BS represent the soil microcosms from Mt. Baicaowa amended without/with salicylate, whereas M and MS refer to the  
685 soil microcosms from Mountain Maoer amended without/with salicylate. “ - ” means samples were not set at the time.

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693 **Fig. 1.** Relative abundance of TRFLP fragments (digested by HaeIII) assigned to *Burkholderial*  
 694 (A), *Burkholderia2* (B), *Terrimonas* (C), *Oxalobacteraceae* (D) against the buoyant density  
 695 gradients in B, BS, M and MS treatments. The bacterial DNA were extracted from B (microcosms  
 696 from Mt. Baicaowa soil) and BS (microcosms from Mt. Baicaowa soil amended with salicylate)  
 697 after 7, 14 and 28 days, and M (microcosms from Mt. Maoer soil) and MS (microcosms from Mt.  
 698 Maoer soil amended with salicylate) after 14, 28 and 42 days. Figure symbols:  $\square$   $^{13}\text{C}$ -BaP;  $\blacksquare$   
 699  $^{12}\text{C}$ -BaP.  $\star$  shows the fractions used for sequencing of partial PAH-RHD $\alpha$  and 16S rRNA genes  
 700 involved in BaP degradation in all the treatments.

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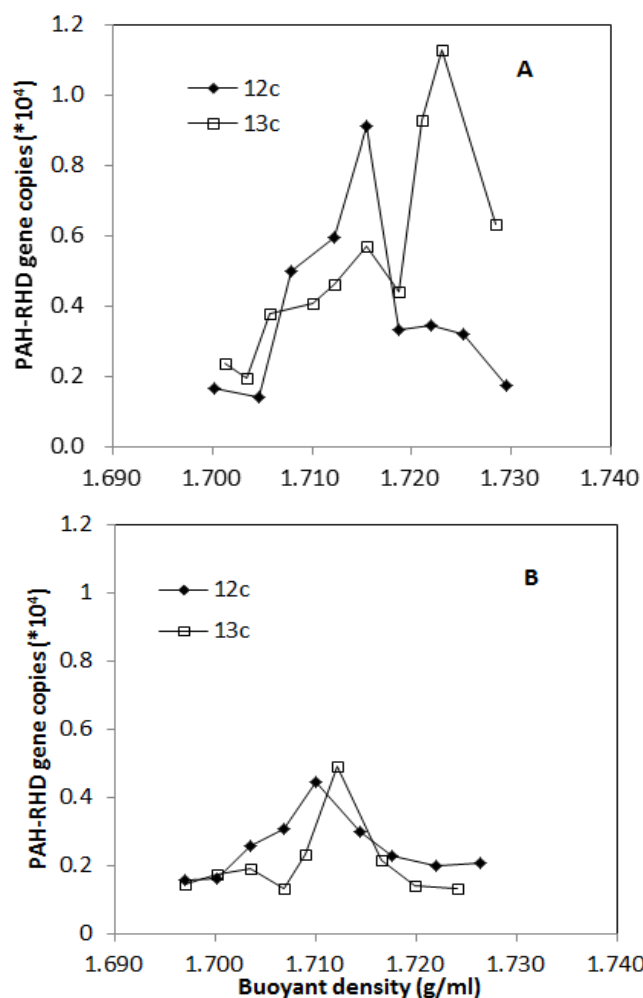
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**Fig. 2.** Phylogenetic tree of PAH-RHDα-M genes from MS (microcosms from Mt. Maoer soil amended with salicylate) and M (microcosms from Mt. Maoer soil without salicylate) treatments along with the closest matches in GenBank, constructed with MEGA 5.0 software using the neighbor-joining method.



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713 **Fig. 3.** PAH-RHD $\alpha$ -M gene copies in ultracentrifugation fractions from <sup>13</sup>C-BaP and <sup>12</sup>C-BaP  
714 amended microcosms determined by qPCR. A and B represent the microcosms from Mt. Maer  
715 soil with/without salicylate addition. Figure symbols: □ <sup>13</sup>C-BaP (~52% BaP degraded); ■  
716 <sup>12</sup>C-BaP (~42% BaP degraded).

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