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# Coupling magnetic-nanoparticle mediated isolation (MMI) and stable isotope probing (SIP) for identifying and isolating the active microbes involved in phenanthrene degradation in wastewater with higher resolution and accuracy

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ABSTRACT

Stable isotope probing (SIP) is a cultivation-independent approach identifying the functional microbes in their natural habitats, possibly linking their identities to functions. DNA-SIP is well-established but suffers from the shift of $^{12}$C-DNA into the heavy DNA ($^{13}$C-DNA) fraction, which significantly reduces the resolution and accuracy. In this study, we coupled magnetic-nanoparticle mediated isolation (MMI) and DNA-SIP, namely MMI-SIP, to identify the active microbes involved in phenanthrene degradation from PAH-contaminated wastewater. Microbes affiliated to *Pseudomonas* and *Sphingobium* were responsible for *in situ* phenanthrene metabolism from the SIP results, and *Pigmentiphaga* was only unraveled for phenanthrene degradation in the MMI and MMI-SIP microcosms. MMI-SIP also significantly increased the enrichment of the above microbes and genes encoding the alpha subunit of the PAH-ring hydroxylating dioxygenase (PAH-RHD$_\alpha$) in the heavy DNA fractions. Our findings suggest that MMI-SIP is a powerful tool, with higher resolution and accuracy, to distinguish the active microbes involved in phenanthrene metabolism in the wastewater, provide a more precise map of functional microbial communities, and offer suggestions for effective management for wastewater treatment plants.



## 1. Introduction

Owing to the rapid economic, industrial and agricultural development, numerous industrial and agricultural chemicals have been released into the environment, causing serious water pollution (Brebbia and Anagnostopoulos, 1991; Colin and Michael, 2012; Kozaki et al., 2017). Various recalcitrant compounds in the effluent are a major source of toxic pollutants to aquatic and nearby environments, causing one of the major concerns over long-term sustainability of the environment (Adenuga et al., 2006; Verma and Samanta, 2017). Of them, polycyclic aromatic hydrocarbons (PAHs) are carcinogenic (Song et al., 2016). Several endeavors have been made to resolve the water pollution and biological treatment is a commonly used method for treating contaminated wastewater (Li et al., 2017a). However, only a small proportion of microbes in the wastewater communities have the direct functions of eliminating PAHs (Li et al., 2017b), it is therefore important to identify and isolate those functional microbes involved in the degradation of PAHs, which can help explore their metabolic characteristics and enhance their performance in wastewater treatment plants. Nevertheless, the majority of microorganisms present in the natural environment are yet-to-be-cultivated, questioning effective approaches to investigate their physiological features and functions (Jiang et al., 2018; Rappé and Giovannoni, 2003).

It is always a great challenge when using traditional approaches to isolate an axenic culture with defined and artificial medium to reveal the *in situ* ecological functions of yet-to-be-cultivated microorganisms. Cultivation-independent methods, which enrich or

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isolate the functional microbes in their natural habitats, have been developed for identifying yet-to-be-cultivated bacteria in the last few decades. High-throughput sequencing gives an unprecedented view of the diversity of microbial communities in environmental samples by providing higher resolution of microbial taxa compared with conventional cloning techniques (Gutierrez, 2011). However, it cannot directly identify the metabolism or functions of the microorganisms. Stable isotope probing (SIP) is an another cultivation-independent technique to assess metabolic responses and provides the opportunity to link the identities of microorganisms to their functions in their natural habitats (Dumont and Murrell, 2005; Radajewski et al., 2000). This method introduces substrates labeled with stable isotopes (*e.g.*, $^{13}C$ or $^{15}N$) to monitor their flow from substrates to biomolecules and identify the functional microbes. SIP relies on the incorporation of stable isotopes into different intracellular components (DNA, RNA, or proteins) of microbial cells (Dumont and Murrell, 2005), which are able to be separated to identify the isotope incorporated fraction, namely DNA-SIP, RNA-SIP and protein-SIP. DNA is more stable than RNA and is the most informative biomarker, providing the highest taxonomical resolution and capable of identifying a broad spectrum of microorganisms involved in a specific metabolic process in the environment (Dumont and Murrell, 2005; Jiang et al., 2018). Although RNA- and protein-SIP is more sensitive than DNA-SIP (Lueders et al. 2004, 2016; Manefield et al. 2002, 2007), they are questioned for lower taxonomic resolution in comparison with DNA-SIP (Lueders et al., 2016). Accordingly, DNA-SIP is most frequently used in environmental microbiology and ecology (Jiang et al., 2018). However, DNA-SIP suffers from the shift of $^{12}C$-DNA into the heavy DNA ($^{13}C$-DNA) fraction, which significantly reduces the resolution and accuracy in distinguishing the functional microbes, particularly in complex microbial communities (Li et al., 2017a).

Magnetic-nanoparticle mediated isolation (MMI) is a recently developed method that can separate living active functional microbes from complex microbiota without substrate labeling (Zhang et al., 2015). This method magnetizes all the microbes of the whole microbial community with biocompatible magnetic nanoparticles (MNPs), and then separates the active microbes which gradually divide and ultimately lose their magnetic attraction from the non-dividing bacteria maintaining magnetism via external magnet (Lin et al., 2015). To date, MMI has been used successfully to separate and characterize the active degraders of cellulose (Zhao et al., 2016), alkanes (Wang et al., 2016) and phenol (Zhang et al., 2015), helping in unraveling their physiological and metabolic characteristics. Although DNA-SIP was used to confirm the functions of the microbes isolated *via* MMI (Zhang et al., 2015), no previous study has coupled MMI with DNA-SIP for identifying the functional microbes simultaneously, which might provide a new approach with higher resolution and accuracy to eliminate the potential interference from $^{12}C$-DNA in the heavy DNA fraction.

To address this issue, we developed a novel cultivation-independent technique coupling MMI and SIP in this study, namely MMI-SIP, to identify and isolate the active microbes involved in phenanthrene degradation from PAH-contaminated wastewater. Phenanthrene was chosen as the target chemical since it is a model PAH compound with the fused-ring structure in an angular fashion and has ubiquity in nature. The microbes responsible for *in situ* assimilation of carbon from phenanthrene in the wastewater microcosm were successfully characterised using DNA-SIP, MMI and MMI-SIP, respectively. High-throughput sequencing and quantitative polymerase chain reaction (qPCR) were applied for a more complete understanding of the bacterial communities and functional genes contributing to phenanthrene degradation. The results showed that MMI-SIP significantly increased the resolution in distinguishing the active microbes involved in phenanthrene degradation compared to SIP and MMI individually, and provided a more accurate and complete map of the active phenanthrene degrading community. Our findings suggest that MMI-SIP is a powerful tool to identify and isolate the active microbes responsible for assimilating carbon from phenanthrene from a complex microbial community with higher resolution and accuracy, and is potentially feasible to distinguish and separate functional microbes responsible for other organic chemical metabolism or carbon cycle in natural environment.

## 2. Materials and methods

### 2.1. Sample collection

Wastewater sample was collected from an untreated industrial wastewater at an oil refinery located in Shandong Province (37°52′N, 118°25′E), China. This wastewater was directly discharged into river nearby. Accordingly, identifying the active microbes in wastewater responsible for phenanthrene degradation was important for designing the wastewater treatment plant. After transport to the laboratory in iceboxes, portions of the samples were stored at −20 °C for subsequent DNA extraction, and the remaining samples were immediately stored at 4 °C before SIP, MMI and MMI-SIP experiments. The contents of PAHs in the wastewater were quantified using gas chromatograph-mass spectrometry (GC-MS) as described in Section 2.7 and listed in Table S1 (Electronic Supporting Information, ESI).

### 2.2. MNPs synthesis and functionalization of wastewater

The synthesis of MNPs was carried out according to previous instructions (Zhang et al. 2011, 2015). After wastewater samples (~1.0 L) were centrifuged at 3000 rpm for 10 min, the cell pellets were re-suspended in the same volume of deionized water and then mixed with 50 mL of biocompatible MNPs stock solution. After shaking (150 rpm) for 20 min at room temperature, the MNPs-functionalized bacteria were separated from the aqueous phase by a permanent magnet. After removing the residual magnetic free cells, the harvested cell pellets were further washed with filter-sterilized wastewater for three times and finally re-suspended in 1.0 L filter-sterilized wastewater.

### 2.3. SIP, MMI and MMI-SIP microcosms

The SIP microcosms were constructed in a 150-mL serum bottle containing 50 mL of the original wastewater. Unlabeled phenanthrene (99%; Cambridge Isotope Laboratories, Inc., USA) or $^{13}C$-labeled phenanthrene ($^{13}C_{14}$-phenanthrene, 99%; Cambridge Isotope Laboratories, Inc., USA) was added to the above bottles with a rubber stopper and an aluminium cap using a gas-tight syringe at a final concentration of 10 mg/L. The sterile control was prepared by adding unlabeled phenanthrene in filter-sterilized wastewater to evaluate the phenanthrene degradation under abiotic conditions. Each treatment was carried out in triplicates. All the microcosms were incubated in the dark with shaking at 120 rpm and room temperature (~25 °C) as previously described (Li et al., 2017a).

The MMI microcosms were prepared by adding unlabeled phenanthrene (10 mg/L) into the filter-sterilized wastewater with MNPs-functionalized bacteria. After 3-day incubation, the active microbes involved in phenanthrene metabolism divided and eventually lost magnetism. By magnetic harvesting, they were separated from the inert microbes and remained in aqueous phase as magnetic-free cell (MFC) suspension. Portions of the MFC suspensions were either stored at −20 °C for subsequent DNA

extraction or added with unlabeled phenanthrene to explore their ability to degrade phenanthrene. To prepare the MMI-SIP microcosms, the 50 mL of collected MFCs were transferred into a 150-mL serum bottle, added with unlabeled or $^{13}$C-phenanthrene to a final concentration of 10 mg/L. The experimental procedures and incubation conditions were the same as the SIP microcosms.

In total, five biotic treatments were carried out as: $^{12}$C_SIP (original wastewater with $^{12}$C-phenanthrene), $^{12}$C_MMI-SIP (MFC with $^{12}$C-phenanthrene), $^{13}$C_SIP (original wastewater with $^{13}$C-phenanthrene), $^{13}$C_MMI-SIP (MFC with $^{13}$C-phenanthrene), and MMI (biocompatible-MNPs functionalized bacteria with $^{12}$C-phenanthrene) (Fig. 1). From the analysis of residual phenanthrene, phenanthrene degradation efficiency achieved over 60% on Day 3 and almost 100% on Day 6 (Table S2). To collect bacterial cells with a higher stable-isotope labelling ratio and avoid cross-feeding, all the samples were taken on Day 3 for DNA extraction.

### 2.4. Nucleic acid extraction, ultracentrifugation and sequencing

The total nucleic acids were extracted from each sample of all the treatments. Proportions of DNA extracted from all the microcosms were directly sequenced. The rest DNA from $^{12}$C- and $^{13}$C-phenanthrene amended microcosms were subjected to ultracentrifugation, fractionation and high-throughput sequencing of each

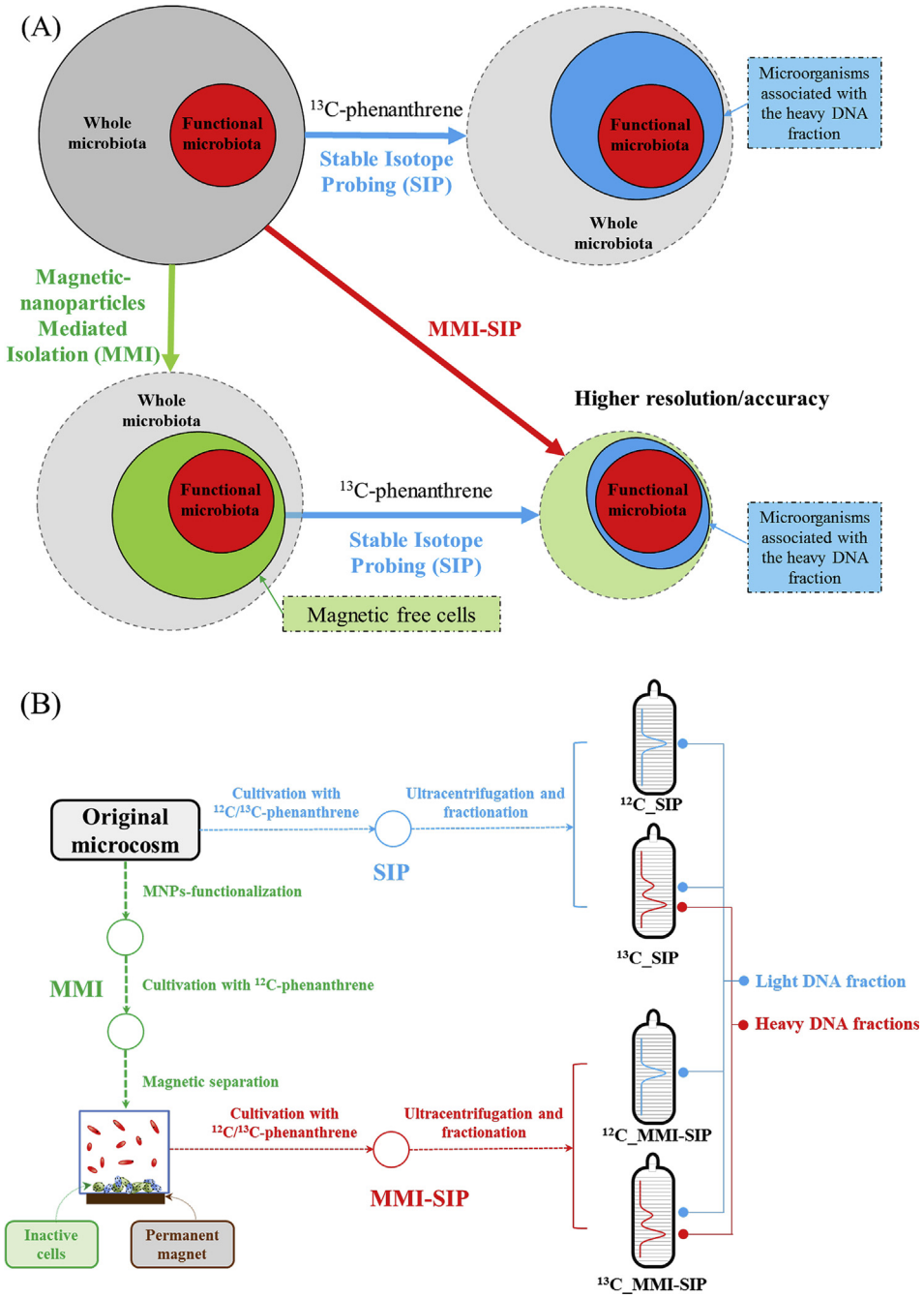


**Fig. 1.** (A) Principles of stable isotope probing (SIP), magnetic-nanoparticles mediated isolation (MMI) and MMI-SIP in identifying and separating the functional microbes from the whole microbiota. (B) Schematic protocols of separating and identifying the functional microbes from the whole microbiota *via* MMI-SIP, comparing to SIP and MMI alone.

fraction according to previously described methods (Song et al., 2016). The hypervariable V4 region of bacterial 16S rRNA gene fragments was amplified using the 515f/806r primer set (Table 1), sequenced using 2 × 250 bp PE technology on an Illumina MiSeq sequencer, and analyzed by MOTHUR (Caporaso et al., 2010; Schloss et al., 2009). In the SIP and MMI-SIP microcosms, the active microbes involved in phenanthrene metabolism were identified by OTUs enriched in the $^{13}$C-DNA fractions from the $^{13}$C_SIP comparing to the $^{12}$C_SIP, and the $^{13}$C_MMI-SIP comparing to the $^{12}$C_MMI-SIP, respectively. For the MMI treatments, the OTUs enriched in the MFC suspension comparing to the whole microbial community were responsible for *in situ* phenanthrene degradation. All the details for nucleic acid extraction, ultracentrifugation and sequencing are provided in ESI.

### 2.5. Detection of genes encoding the alpha subunit of the PAH-ring hydroxylating dioxygenase (PAH-RHD$_\alpha$)

PAH-RHD$_\alpha$ gene is usually used as the biomarker to quantify the PAH degrading bacteria (Cebron et al., 2008). The PAH-RHD$_\alpha$ genes in the MFC suspension and the heavy DNA fractions from the $^{13}$C_SIP or $^{13}$C_MMI-SIP microcosms were amplified using two primer sets for Gram-positive (GP, 642f/933r) and Gram-negative (GN, 610f/911r) degraders, respectively (Table 1) (Cebron et al., 2008). Gradient PCR and the amplification reactions were performed as described previously (Li et al., 2017a). In the present study, only one strong and specific amplicon was produced with PAH-RHD$_\alpha$ GN primer set and selected for following analysis. The PCR products were gel-purified using a gel extraction kit (D2500-01, Omega Bio-tek, Norcross, GA, USA). Purified amplicons were cloned and sequenced according to Jiang's method (Jiang et al., 2015). The phylogenetic dendrogram was prepared using the method described above. The GenBank accession number for the partial PAH-RHD$_\alpha$ GN gene sequence is MG806915. The partial 16S rRNA gene sequences obtained are available in GenBank with the following accession numbers: MG800821 for OTU_32, MG800822 for OTU_16, MG800823 for OTU_53, MG800824 for OTU_35, and MG800825 for OTU_10.

### 2.6. Quantitative PCR (qPCR)

The abundances of bacterial 16S rRNA and PAH-RHD$_\alpha$ GN genes in each treatment were determined by qPCR with a bacterial universal primer pair of Bac519F/Bac907R and the PAH-RHD$_\alpha$ GN primer pair of 610f/911r, respectively (Table 1). The qPCR reactions were performed following the process described in our previous work (Li et al., 2017b). Two standard curves were obtained by producing a 10-fold serial dilution of plasmid pGEM-T Easy Vector sequences ($10^2$-$10^8$ copies, Promega) containing the 16S rRNA and PAH-RHD$_\alpha$ GN genes, respectively. At the end of the qPCR, a melt curve was obtained by heating from 60 to 95 °C at a rate of 0.2 °C/cycle.

**Table 1**
PCR primers used for 16S rRNA and PAH-RHD$_\alpha$ genes.

| Target | Primer | Sequence (5′-3′) |
|---|---|---|
| 16S rRNA | 515f | GTGCCAGCMGCCGCGGTAA |
| | 806r | AACGCACGCTAGCCGGACTACVSGGGTATCTAAT |
| | Bac519f | CA GCMGCCGCGGTAANWC |
| | Bac907r | CCGTCAATTCMTTTRAGTT |
| PAH-RHD$_\alpha$ | 610f | GAGATGCATACCACGTKGGTTGGA |
| | 911r | AGCTGTTGTTCGGGAAGAYWGTGCMGTT |
| | 642f | CGGCGCCGACAAYTTYGTNGG |
| | 933r | GGGGAACACGGTGCCRTGDATRAA |

### 2.7. Chemical analysis

Phenanthrene in wastewater from each microcosm (Day 0, 3 and 6 after incubation) was analyzed by gas-chromatograph (model 7890, Agilent, Santa Clara, CA, USA), using a capillary column (DB-5MS, 30 m, 0.25 mm, 0.25 μm) and a mass spectrometer (MSD, model 5975, Agilent), as previously described (Li et al., 2017a). Briefly, the water samples were spiked with 1000 ng deuterated PAHs and extracted twice with dichloromethane. After purification with a silica-gel/alumina column (8 mm i.d.), the eluent was concentrated to approximately 50 μL using a gentle stream of $N_2$, and 1000 ng hexamethylbenzene was added as an internal standard to all the samples before the instrumental analysis. The concentrations of deuterated PAHs, PAHs standards and internal standard are listed in Table S3.

## 3. Results

### 3.1. Active microbes involved in phenanthrene degradation as revealed by SIP

The phenanthrene degradation efficiencies in $^{12}$C_SIP and $^{13}$C_SIP microcosms were shown in Table S2. The residual phenanthrene in the sterile control exhibited less decrease than those in the biotic treatments. Residual phenanthrene was 32−37% in SIP microcosms after 3-day incubation, and no significant difference ($p > 0.05$) was observed between the $^{12}$C_SIP and $^{13}$C_SIP treatments.

The whole community structure at genus level showed only slight difference between $^{12}$C_SIP and $^{13}$C_SIP treatments (Fig. 2). The dominant bacteria (>5% total abundance) included members of the genera *Kaistobacter*, *Rhodanobacter*, *Sphingobacterium*, unclassified *Comamonadaceae*, *Enterobacteriaceae* and *Xanthomonadaceae*.

The DNA in the fractions with buoyant densities (BDs) of 1.7198 and 1.7556 g/mL were selected as 'light' and 'heavy' DNA fractions, respectively, from both $^{12}$C_SIP and $^{13}$C_SIP microcosms, which was supported by our concentration-BD curves (Fig. S1). Additionally, the active microbes responsible for $^{13}$C assimilation were analyzed by comparing the relative abundances of OTUs in the $^{12}$C_SIP and $^{13}$C_SIP treatments from each fraction. As shown in Fig. S2A, the relative abundances of OTU_32 and OTU_16 at higher BD (1.7556 g/mL) were higher in the $^{13}$C_SIP microcosms than those in the $^{12}$C_SIP samples. In contrast, no such enrichment or similar trend was detected in the DNA fractions with lower BD. The results indicated that microorganisms represented by these two OTUs played a primary role in phenanthrene degradation. Fig. 3 shows the phylogenetic information for the identified active microbes involved in phenanthrene metabolism. OTU_32 belonging to the genus *Pseudomonas* (phylum *Proteobacteria*, class *Gammaproteobacteria*, order *Pseudomonadales*, family *Pseudomonadaceae*) exhibits 100% similarity to the partial 16S rRNA gene sequence of *Pseudomonas* strains such as uncultured *Pseudomonas* sp. clone OTU 1378 (KR832662.1), *Pseudomonas nitroreducens* AIMST Ehe20 (JQ312053.1) and *Pseudomonas* sp. DN30 (AY581447.1), and forms a subclade with a high bootstrap value of 99. OTU_16 is assigned to the genus *Sphingobium* (phylum *Proteobacteria*, class *Alphaproteobacteria*, order *Sphingomonadales*, family *Sphingomonadaceae*) and shares 99% similarity with *Sphingobium* sp. dBA-3 (GQ176409.1).

### 3.2. Active microbes responsible for phenanthrene metabolism as revealed by MMI

The MMI microcosms had a similar phenanthrene degradation performance as the SIP microcosms (Table S2). The active microbes involved in phenanthrene metabolism were evaluated by

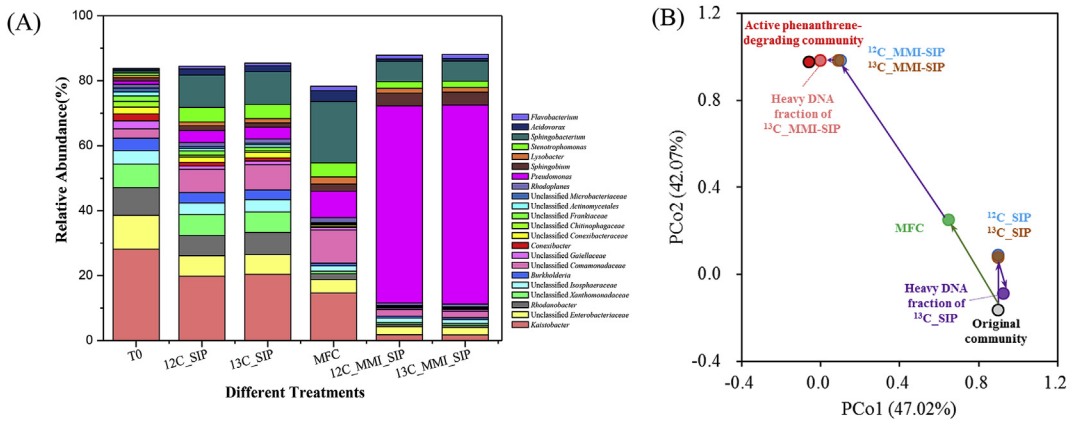


**Fig. 2.** (A) Relative abundance of 16S rRNA gene defined taxa by genus in the $^{12}$C_SIP, $^{13}$C_SIP, MFC, $^{12}$C_MMI-SIP, and $^{13}$C_MMI-SIP microcosms before ultracentrifugation. The selected taxa have a minimal relative abundance greater than 1%. T0 represents the original microbial community in wastewater. (B) PCoA score plots (Bray-Curtis matrix) illustrating the distance between microbial communities of the $^{12}$C_SIP, $^{13}$C_SIP, MFC, $^{12}$C_MMI-SIP, $^{13}$C_MMI-SIP, the heavy DNA fractions of the $^{13}$C_SIP and $^{13}$C_MMI-SIP. Principal coordinate 1 (PCo1) and 2 (PCo2) explain 47.02% and 42.07% of the total variance in microbial community structure.

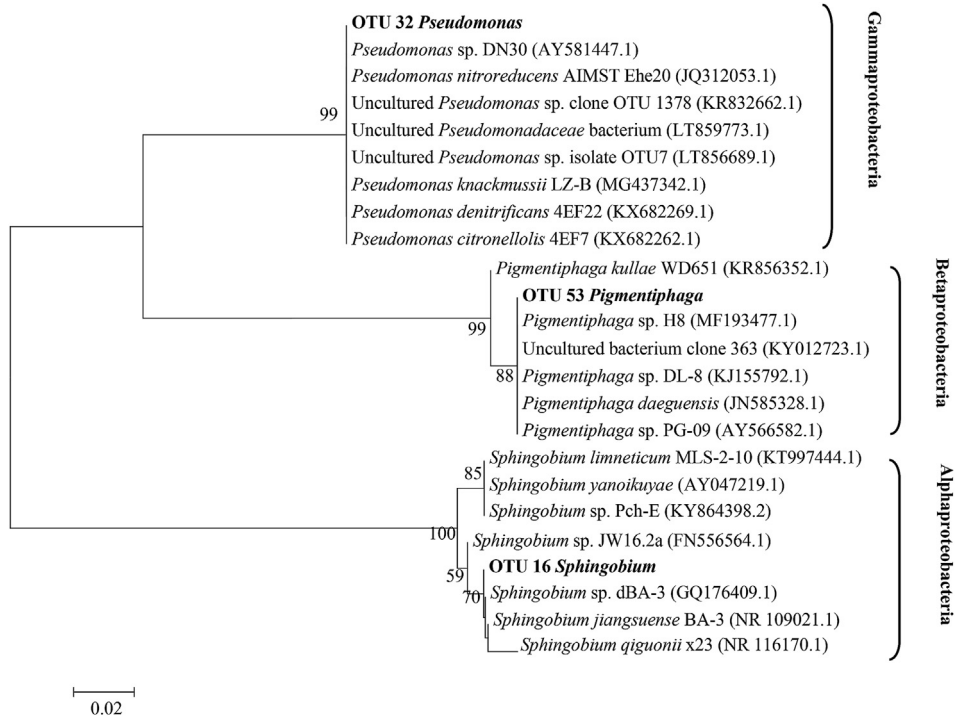


**Fig. 3.** Phylogenetic tree of the identified OTUs responsible for *in situ* phenanthrene degradation. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of the microorganisms corresponding OTU_32, OTU_16, OTU_53 and their representatives of some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) > 50% are shown at the branch points. Bar 0.02 substitutions per nucleotide position.

comparing the ratio of the relative abundances of top 100 OTUs in the MFC suspension to those in the inert microbial community. Five OTUs were enriched and their enrichment ratios were 2.28 (OTU_16), 4.92 (OTU_32), 4.45 (OTU_53), 2.34 (OTU_5) and 2.81 (OTU_10), respectively (Table S4 and Fig. S3). Among them, OTU_16 and OTU_32 were also identified as the active microbes responsible for assimilating carbon from phenanthrene in the $^{13}$C_SIP microcosms, and our MMI results further confirmed their involvement in phenanthrene metabolism. Of three new OTUs (OTU_53, OTU_5 and OTU_10), OTU_53 is assigned to the genus *Pigmentiphaga* (phylum *Proteobacteria*, class *Betaproteobacteria*, order *Burkholderiales*, family *Alcaligenaceae*), and shares 99% similarity with *Pigmentiphaga* sp. H8 (MF193477.1) (Fig. 3). OTU_10 belongs to the genus *Sphingobacterium* (phylum *Bacteroidetes*, class *Sphingobacteriia*, order *Sphingobacteriales*, family *Sphingobacteriaceae*) and OTU_5 is unclassified *Comamonadaceae* (phylum *Proteobacteria*, class *Betaproteobacteria*, order *Burkholderiales*). As these microorganisms represented by OTU_53, OTU_5 and OTU_10 were not enriched in the heavy DNA fraction of the $^{13}$C_SIP treatments (Figs. S2 and S4), their roles in phenanthrene degradation were suspected. They might be the active microbes involved in phenanthrene degradation but not distinguished in the SIP microcosms due to their extremely low abundance, or microbes utilizing other carbon sources in wastewater with higher growth rate than other bacteria.

### 3.3. Active microbes involved in phenanthrene metabolism as revealed by MMI-SIP

The phenanthrene degradation in the $^{12}$C_MMI-SIP and $^{13}$C_MMI-SIP treatments was similar (Table S2). The residual phenanthrene after 3 days (14–18%) was significantly lower ($p < 0.05$) than those in the SIP and MMI microcosms, achieving 18–19% increase of phenanthrene degradation efficiency.

Slight difference was observed in the indigenous microbial communities between the $^{12}$C_MMI-SIP and $^{13}$C_MMI-SIP treatments, consistent with our results in the SIP microcosms (Fig. 2). The composition of the whole microbial communities behaved significantly different between SIP and MMI-SIP microcosms. In the MMI-SIP microcosms, the relative abundance of the genus *Pseudomonas* (61.1%) increased and was much higher than those in the SIP microcosms (3.71%), whereas the relative abundances of the genera *Kaistobacter*, *Rhodanobacter*, *Sphingobacterium*, unclassified *Comamonadaceae*, *Enterobacteriaceae* and *Xanthomonadaceae* were significantly lower (Table S5).

Three OTUs enriched in the heavy DNA fractions of the $^{13}$C_MMI-SIP microcosms included two identified ones in the SIP microcosms (OTU_32, OTU_16) and one (OTU_53) in the MMI treatments (Fig. S2B). Similarly, the relative abundances of OTU_32, OTU_16 and OTU_53 in the heavy DNA fractions of the $^{13}$C_MMI-SIP treatments (41.1%, 13.6% and 2.75%, respectively) were significantly higher than those in the $^{12}$C_MMI-SIP microcosms (15.8%, 2.38%, and 0.28%, respectively). Meanwhile, OTU_5 and OTU_10, which were enriched in MMI treatments, were not incorporated with $^{13}$C-phenanthrene assimilation owing to their low enrichment in the heavy DNA fraction (Fig. S5), suggesting that microorganisms represented by these two OTUs were not the active bacteria involved in phenanthrene degradation.

### 3.4. Differences in microbial community and active microbes involved in phenanthrene metabolism among the MFC and the heavy DNA fractions of the $^{13}$C_SIP and $^{13}$C_MMI-SIP microcosms

The microbial community structure was significantly different between the MFC suspension and the heavy DNA fractions of the $^{13}$C_SIP microcosms. In the MFC suspension, the relative abundances of the genera *Pseudomonas*, *Sphingobacterium* and unclassified *Comamonadaceae* increased and were significantly higher than those in the heavy DNA fractions of the $^{13}$C_SIP microcosms (Fig. S6), whereas the relative abundance of the genus *Kaistobacter* was much lower (Table S6). In the MMI-SIP microcosms, the relative abundances of the genera *Pseudomonas* and *Sphingobium* remarkably increased, whereas the relative abundances of the genera *Kaistobacter*, *Sphingobacterium*, unclassified *Comamonadaceae*, *Enterobacteriaceae* and *Xanthomonadaceae* behaved oppositely (Table S6).

The microorganisms represented by OTU_32, OTU_16 and OTU_53 were confirmed for phenanthrene metabolism. After 3 days, their relative abundances were 1.52% (OTU_32), 0.51% (OTU_16) and 0.02% (OTU_53) in the heavy DNA fractions of the $^{13}$C_SIP microcosms, 5.18% (OTU_32), 1.03% (OTU_16) and 0.16% (OTU_53) in the MFC suspension, and 41.13% (OTU_32), 13.56% (OTU_16) and 2.75% (OTU_53) in the heavy DNA fractions of the $^{13}$C_MMI-SIP microcosms. Accordingly, these active microbes involved in phenanthrene metabolism were significantly more enriched in the $^{13}$C_MMI-SIP microcosms than both MMI and $^{13}$C_SIP treatments (Fig. 4A). Moreover, the above three OTUs were better enriched in the heavy DNA fractions of $^{13}$C_MMI-SIP microcosms than those of $^{13}$C_SIP treatments (Fig. 4B). Accordingly, MMI alone had a higher resolution in identifying the active microbes involved in phenanthrene metabolism comparing to SIP (5 OTUs *vs* 2 OTUs), but microorganisms represented by OTU_10 and OTU_5 did not participate in phenanthrene metabolism owing to the poor enrichment in the heavy DNA fraction from $^{13}$C_MMI-SIP microcosms. These results suggested that MMI-SIP could identify the active microbes responsible for *in situ* phenanthrene metabolism with higher accuracy comparing to SIP and MMI.

The microbial structure had significant changes across the $^{12}$C_SIP, $^{13}$C_SIP, MMI, $^{12}$C_MMI-SIP, $^{13}$C_MMI-SIP, MFC, and the heavy DNA fractions of the $^{13}$C_SIP and $^{13}$C_MMI-SIP microcosms. PCoA score plot (Fig. 2B) illustrated that all the bacterial communities exhibited a dramatic shift from the location of original wastewater community. The groups of $^{12}$C_SIP, $^{13}$C_SIP and the heavy DNA fractions of $^{13}$C_SIP were located closer to the original wastewater than the active microbial community responsible for phenanthrene degradation. In contrast, the group of the MFC suspension shifted directly towards the active community involved in phenanthrene degradation, indicating that MMI could separate the active microbes involved in phenanthrene metabolism. Further coupling with SIP, the community structures from both $^{12}$C_MMI-SIP and $^{13}$C_MMI-SIP were much closer to the active community involved in phenanthrene degradation. Moreover, the microbial community structure of the heavy DNA fraction from the $^{13}$C_MMI-SIP was located closest to the active microbial community responsible for phenanthrene degradation, indicating that MMI-SIP can provide a more precise map of the active microbial community responsible for phenanthrene degradation from complex microbiota than SIP or MMI individually.

### 3.5. Occurrence and quantification of PAH-RHD$_\alpha$ genes involved in phenanthrene metabolism

The PAH-RHD$_\alpha$ GN genes were analyzed in the MFC, the heavy DNA fractions of the $^{13}$C_SIP and $^{13}$C_MMI-SIP microcosms in the present study. In all the microcosms, the PAH-RHD$_\alpha$ gene sequences are identical and share 98% similarity with that of uncultured *Pseudomonas* sp. clone Berre6A3B-3 (AM743143.1) (Fig. S8).

The PAH-RHD$_\alpha$ genes in the SIP and MMI-SIP treatments were quantified against each density-resolved fraction (Fig. S9). A remarkable enrichment in the heavy DNA fractions (highlighted in grey) was observed in both $^{13}$C_SIP and $^{13}$C_MMI-SIP treatments, indicating that the PAH-RHD$_\alpha$ genes were labeled with the assimilated $^{13}$C. Accordingly, the PAH-RHD$_\alpha$ genes detected in the heavy DNA fractions of the $^{13}$C-phenanthrene microcosms were associated with phenanthrene metabolism. In addition, the copy number of PAH-RHD$_\alpha$ genes was $0.32 \times 10^4$, $1.09 \times 10^4$ and $2.46 \times 10^4$ copies/ng DNA in the heavy DNA fractions of the $^{13}$C_SIP microcosms, MFC suspension and the heavy DNA fractions of the $^{13}$C_MMI-SIP microcosms (Fig. 5A). It indicated that MMI-SIP and MMI could enrich PAH-RHD$_\alpha$ genes in the PAH-contaminated wastewater *via* separating the active microbes involved in phenanthrene metabolism. Moreover, the enrichment ratio of PAH-RHD$_\alpha$ gene was 8.82 in the heavy DNA fractions of the MMI-SIP microcosms, remarkably higher than that in the SIP microcosms (3.54, Fig. 5B).

## 4. Discussion

Water is a fundamental source for human subsistence, and the increasing water pollution is therefore an imperative problem severely affecting human health worldwide (Sevda et al., 2018). Conventional biological treatment is considered as a cost-effective and eco-friendly approach for treating PAH-contaminated wastewater (Li et al., 2018), but suffering from a critical drawback that the majority of microbes responsible for *in situ* PAHs degradation are yet-to-be-cultivated. New approaches are required to properly

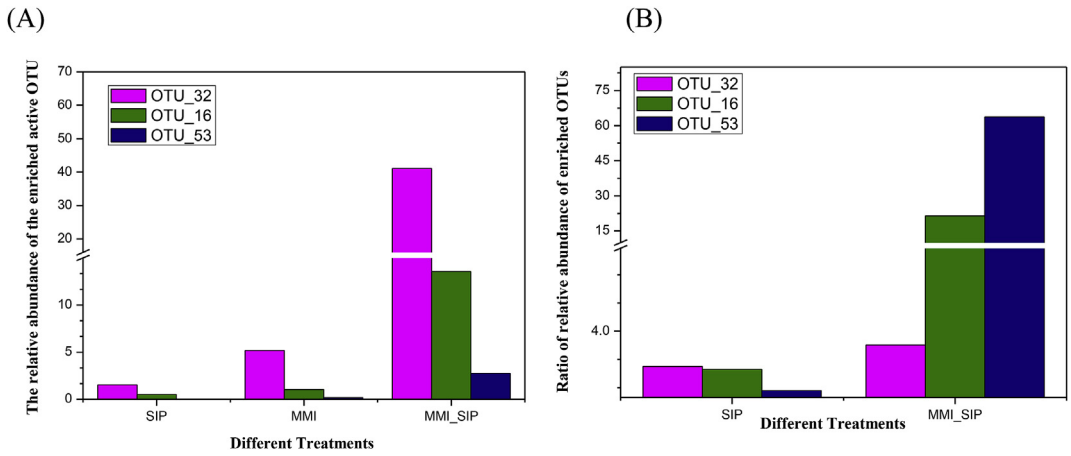


**Fig. 4.** (A) The relative abundance of the enriched OTUs in the MFC and the heavy DNA fractions of the $^{13}$C_SIP and $^{13}$C_MMI-SIP microcosms. (B) Ratio of relative abundance of the enriched OTUs detected in the heavy DNA fractions of the $^{13}$C-phenanthrene microcosms to those in the light DNA fractions.

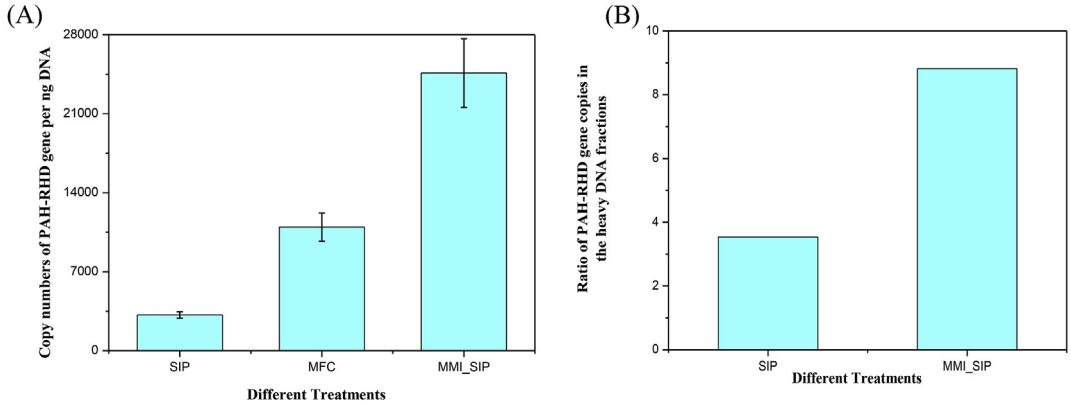


**Fig. 5.** (A) Copy numbers of PAH-RHD$_\alpha$ gene in the MFC and the heavy DNA fractions of the $^{13}$C_SIP and $^{13}$C_MMI-SIP microcosms. (B) Ratio of PAH-RHD$_\alpha$ gene copies in the heavy DNA fractions of the $^{13}$C-phenanthrene microcosms to those of the $^{12}$C-phenanthrene microcosms.

estimate the diversity and functions of the active microbes involved in PAHs metabolism in wastewater.

SIP relies on the incorporation of a stable isotope-enriched substrate into the active microorganisms. As DNA is the most informative taxonomic biomarkers (Dumont and Murrell, 2005), DNA-SIP is increasingly applied in attempts to link the identities of microorganisms to their functions in natural habitats. Heavy DNA labeled with stable isotope could be separated from unlabeled light DNA by density-gradient centrifugation (Dumont and Murrell, 2005). They are further used as templates to amplify the target genes for bacterial identity (16S rRNA genes) or functions (functional genes) (Neufeld et al., 2007b). DNA-SIP has been successfully applied to study the active populations with specific functions in environment samples, such as PAH-degrading bacteria (Jiang et al., 2015; Li et al., 2017b; Song et al., 2016) and methane-oxidizing microbes (Morris et al., 2002). It shows a better prospect recently when properly coupled with metagenomic analysis (Chemerys et al., 2014; Ziels et al., 2018). However, DNA-SIP has an inevitable limitation that the light $^{12}$C-DNA always shifts into the heavy $^{13}$C-DNA fraction, which significantly reduces the resolution and accuracy in distinguishing the functional microbes, particularly in complex microbial communities (Li et al., 2017a). Although many previous studies have successfully applied DNA-SIP to investigate the active functional microorganisms (Eyice et al., 2015; Jones et al., 2011), the identified microbes were normally of high abundance (>5%) in the $^{13}$C-DNA heavy fractions (Esson et al., 2016; Eyice et al., 2015; Johnston et al., 2017), and the lowest abundance was above 0.1% (Eyice et al., 2015; Li et al. 2017a, 2017b). It is still not clear whether extremely rare microorganisms (<0.1%) involved in assimilating stable-isotope labeled substrates could be enriched and identified in the heavy DNA fraction.

Alternatively, MMI is a recently developed technique that can effectively separate functional microbes by magnetic fields (Zhang et al., 2015). Unlike DNA-SIP targeting isotope-labeled DNA fragments, MMI does not require substrate labeling and DNA fractionation (Wang et al., 2016). Thus, MMI is considered as a promising tool for identifying the active functional cells in complex environmental media. However, MMI cannot accurately specify the functional microorganisms owing to the contamination from fast-growing bacteria, such as microorganisms represented by OTU_10 and OTU_5 in this study. Accordingly, we hypothesized that MMI coupling with SIP can solve the challenges of either approach mentioned above, as a powerful tool to identify the active functional bacteria from a complex microbial community, with higher resolution and accuracy comparing to either SIP or MMI individually.

In our novel MMI-SIP approach, MMI behaves as a pre-screening tool to separate the active functional microorganisms, raising the enrichment ratio of $^{13}$C-labeled DNA and increasing the resolution to identify the active microbes involved in phenanthrene metabolism (Fig. 1). SIP tracks the flow of stable isotope from $^{13}$C-phenantherene into the active microbes and confirmed their functions

for phenanthrene metabolism (Dumont and Murrell, 2005; Zhang et al., 2015). In the present study, the relative abundance of extremely rare microorganisms (OTU_53, 0.02% in original wastewater) increased to 2.75% in the heavy DNA fractions of the $^{13}$C_MMI-SIP microcosms, proving that MMI-SIP could separate extremely rare microorganisms responsible for *in situ* phenanthrene degradation. Accordingly, MMI-SIP significantly improves the resolution and accuracy for identifying the active functional bacteria. Here, only one consecutive incubation was applied in MMI_SIP treatments to compare the active phenanthrene degraders identified by MMI, SIP and MMI_SIP. Since MMI treatments might enrich phenanthrene-degrading bacteria and increase their assimilation of $^{13}$C-phenantherene, another pre-enrichment with phenanthrene of the wastewater community without MMI is suggested before conducting SIP and it will be improved in our future studies.

It is worth mentioning that MMI-SIP could also provide a more precise map of the active microbial community responsible for phenanthrene degradation than MMI or SIP individually (Fig. 2). Less than 2% of the bacterial populations in the original wastewater were separated in MMI-SIP, suggesting that only a minor proportion of all the microorganisms in wastewater were responsible for *in situ* phenanthrene metabolism. It was concordant with our previous work reporting that only rare species with low abundance were responsible for metabolizing phenanthrene in the whole microbiota (Li et al. 2017a, 2017b). Since SIP could only identify the active microbes involved in phenanthrene metabolism with abundance >0.1% and the enrichment ratio was poor, the microbial community structure of the heavy DNA fractions of the $^{13}$C_SIP microcosms is therefore significantly different from the active microbial community responsible for phenanthrene degradation and shows a huge distance in PCoA score plot (Fig. 2). Although MMI has higher resolution in separating the active functional bacteria, its low accuracy sets the barrier to achieve the precise characterization of the active microbial community responsible for phenanthrene degradation. Our proposed MMI-SIP approach successfully resolves the problems of both SIP and MMI, and the microbial structure of the heavy DNA fraction from the $^{13}$C_MMI-SIP microcosms was very similar to the active microbial community responsible for phenanthrene degradation (Fig. 2).

The active microorganisms responsible for *in situ* phenanthrene degradation are affiliated to *Pseudomonas*, *Sphingobium* and *Pigmentiphaga*. *Pseudomonas* is a well-known PAHs-degrading bacterial lineage and members of this genus contain PAH-RHD$_\alpha$ genes encoding the PAHs degradative pathway (Thomas et al., 2016). *Sphingobium* can also metabolise a wide range of PAHs (Cunliffe and Kertesz, 2006). The degradation of phenanthrene by *Pseudomonas* and *Sphingobium* has been previously reported (Otto et al., 2016). Members of the genus *Pigmentiphaga* possess versatile metabolic capabilities, such as the pathways for degrading benzoxazolinone, acetamiprid and chlorothalonil (Dong et al., 2018). However, limited studies have addressed their ability to metabolise PAHs. The only one example to our knowledge has confirmed that *Pigmentiphaga* were associated with naphthalene and phenanthrene degradation (Jones et al., 2011). Our MMI-SIP results provide direct evidence that members of the genus *Pigmentiphaga* are responsible for *in situ* phenanthrene metabolism in PAH-contaminated wastewater.

Besides higher resolution in identifying the active microbes involved in phenanthrene metabolism, MMI-SIP enriched the relative abundance of PAH-RHD$_\alpha$ genes in the heavy DNA fraction (Fig. 5). The abundance of the RHD$_\alpha$ genes was reported to correlate with PAHs degradation efficiency (Ding et al., 2010), and the temporal changes in the activities of the PAH-degraders bearing PAH-RHD$_\alpha$ genes during PAHs degradation process demonstrated its strong link with bacterial degradation capability (Guo et al., 2017). In the present study, the relative abundance of PAH-RHD$_\alpha$ genes was positively correlated with that of the active microbes involved in phenanthrene metabolism, hinting that they might belong to *Pseudomonas*, *Sphingobium* or *Pigmentiphaga* in the $^{13}$C_MMI-SIP microcosms. Many studies have successfully amplified functional genes using PCR primers from $^{13}$C-DNA (Neufeld et al., 2007a; Uhlik et al., 2009). However, it is difficult to determine the affiliation of the functional gene sequences to 16S rRNA genes (Neufeld et al., 2007a). Future work is suggested to directly link the identities of the active microbes responsible for phenanthrene degradation to their metabolisms via metagenomics and protein-SIP (Jehmlich et al., 2016).

## 5. Conclusions

A novel MMI-SIP method was developed and employed to identify the active microbes involved in phenanthrene metabolism from PAH-contaminated wastewater. Microbes affiliated to *Pseudomonas* and *Sphingobium* were responsible for phenanthrene metabolism from the SIP results. MMI-SIP significantly increased the abundance of *Pseudomonas* and *Sphingobium* in the heavy DNA fractions and found one more *Pigmentiphaga* involved in phenanthrene metabolism. Further evidence was also found from the higher enrichment of PAH-RHD$_\alpha$ genes in the heavy DNA fraction of the $^{13}$C_MMI-SIP than that in the $^{13}$C_SIP microcosms. Our results suggested that MMI-SIP is a reliable cultivation-independent approach to identify functional microbes from complex microbiota, with higher resolution and accuracy comparing to MMI or SIP alone. Additionally, MMI-SIP successfully separated the active phenanthrene-degrading consortium and significantly increased the phenanthrene removal efficiency. Our study indicates that MMI-SIP approach is a successful tool to distinguish the *in situ* active microbes involved in phenanthrene degradation. It might provide a more precise map and new mechanisms of the active microbes involved in the biodegradation of other organic pollutants and help in understanding their ecological roles and influential factors during the microbial degradation processes.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.07.036.

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