Effects of different agricultural wastes on the dissipation of PAHs and the PAH-degrading genes in a PAH-contaminated soil

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HIGHLIGHTS

- Three agricultural wastes were used to a 90-day soil microcosm study.
- Agricultural wastes accelerated dissipation of the aged PAHs in soil microcosms.
- Agricultural wastes with lower C:N ratios eliminated the aged PAHs more quickly.
- Agricultural wastes significantly increased abundances of the PAH-degrading genes.
- Agricultural wastes obviously changed the community structure of PAH-degraders.

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ABSTRACT

Land application of agricultural wastes is considered as a promising bioremediation approach for cleaning up soils contaminated by aged polycyclic aromatic hydrocarbons (PAHs). However, it remains largely unknown about how microbial PAH-degraders, which play a key role in the biodegradation of soil PAHs, respond to the amendments of agricultural wastes. Here, a 90-day soil microcosm study was conducted to compare the effects of three agricultural wastes (i.e. WS, wheat stalk; MCSW, mushroom cultivation substrate waste; and CM, cow manure) on the dissipation of aged PAHs and the abundance and community structure of PAH-degrading microorganisms. The results showed that all the three agricultural wastes accelerated the dissipation of aged PAHs and significantly increased abundances of the bacterial 16S rRNA and PAH-degrading genes (i.e. *pdo1* and *nah*). CM and MCSW with lower ratios of C:N eliminated soil PAHs more efficiently than WS with a high ratio of C:N. Low molecular weight PAHs were dissipated more quickly than those with high molecular weight. Phylogenetic analysis revealed that all of the *nah* and *C120* clones were affiliated within *Betaproteobacteria* and *Gammaproteobacteria*, and application of agricultural wastes significantly changed the community structure of the microorganisms harboring *nah* and *C120* genes, particularly in the CM treatment. Taken together, our findings suggest that the three tested agricultural wastes could accelerate the degradation of aged PAHs most likely through changing the abundances and community structure of microbial PAH degraders.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous organic

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pollutants characterized by high genotoxicity and carcinogenicity, and can enter and enrich in human body through the food chain (Johnsen et al., 2005; Peng et al., 2008; Niepceon et al., 2013). Environmental contamination caused by PAHs therefore poses a serious risk to public health and ecosystem safety (Johnsen et al., 2005; Peng et al., 2008). To date, 16 types of PAHs have been added into the blacklist of priority pollutants by the United States Environmental Protection Agency (USEPA) (Mackay et al., 1992). PAHs are mainly generated in the processes of coke production, petroleum refining and incomplete combustion of organic matters,

and thus high levels of PAHs are frequently found in industrial sites (Johnsen et al., 2005). Aging of PAHs, that is, sequestration of them by soil aggregates, can appear during the contact with soils over time, which makes PAHs more resistant to degradation (Northcott and Jones, 2001; Luo et al., 2012). Therefore, remediation of soils polluted by aged PAHs has become a major issue for environmental scientists in recent years (Alcántara et al., 2008).

A wide variety of physical, chemical and biological approaches have been applied to remediation of soils polluted by organic chemical compounds (Pelaez et al., 2013). Compared to the high cost and potential secondary contamination incurred by physical and chemical methods, bioremediation, especially microbial remediation, is considered as a cost-effective option for cleaning up soils contaminated by PAHs (Jones et al., 2011; Pelaez et al., 2013; Vila et al., 2015). In general, microbial remediation plays an important role in eliminating PAHs from soils through two pathways, which are defined as biostimulation and bioaugmentation (Xu and Lu, 2010; Sayara et al., 2011). Biostimulation involves the modification of soil microbial habitats enabling soil resident bacteria to be more capable of degrading PAHs. For example, additions of organic substrates, oxygen or electron acceptors may enhance the activity of soil indigenous microbial populations to degrade PAHs in situ (Haritash and Kaushik, 2009; Nikolopoulou et al., 2013). By contrast, bioaugmentation is the introduction (or inoculation) of some specific competent PAH-degrading microorganisms including natural microbial strains and genetically engineered variants to enhance the degradation of PAHs in the environment (Sayara et al., 2011; Festa et al., 2016). Numerous studies have reported effective PAH degradation through biostimulation and bioaugmentation strategies (Xu and Lu, 2010), whereas conflicting results existed that the introduced microbial strains could not significantly promote the degradation of PAHs owing to their poor adaptation to the new environmental conditions and the disadvantage in competing with indigenous microbial consortia (Lladó et al., 2013). For instance, Sayara et al. (2011) found that PAH degradation was significantly improved by using compost of the municipal solid waste as materials for biostimulation, while no significant effects were obtained by introducing white-rot fungi *Trametes versicolor*. Wu et al. (2016) compared bioaugmentation using *Acinetobacter* SZ-1 strain and biostimulation using $(\text{NH}_4)_2\text{SO}_4$ and KH_2PO_4 in a petroleum-contaminated soil. It was found that the dissipation of total petroleum hydrocarbons (TPH) and the amounts of cultivable TPH-, alkane- and PAH-degrading microorganisms were higher for biostimulation than for bioaugmentation. Therefore, it is essential to identify the substrates which could be added into soils to promote the degradation of PAHs through the bioremediation pathway.

Nutrient elements such as carbon, nitrogen and phosphorus are necessary for the growth of most microorganisms, and many studies have demonstrated that appropriate carbon sources can promote biodegradation of soil PAHs through introducing the co-metabolic substrates (Sarkar et al., 2005; Lladó et al., 2009). Therefore, addition of organic substrates has been considered as a feasible remediation strategy in aged PAH-contaminated soils harboring abundant active microbial degraders (Antizar-Ladislao et al., 2005; Sayara et al., 2011). Teng et al. (2010) examined the bioremediation effects of three carbon sources (glucose, sodium succinate and starch) on an aged PAH-contaminated soil, and found that addition of carbon sources had a positive effect on the dissipation of phenanthrene and benzo(a)pyrene as well as the cultivable bacterial and fungal counts. Antizar-Ladislao et al. (2005) reported that different ratios of soil/green waste had different effects on removal of PAHs in an aged coal tar soil. Although several studies have showed that microbial degradation of PAHs could be accelerated through adding organic substrates and inorganic

nutrients to contaminated soils (Antizar-Ladislao et al., 2005; Wu et al., 2016), to our knowledge, these studies focused on the amounts of culturable PAH degraders which represent only a minor proportion of the total PAH-degrading populations (Singleton et al., 2005). To better understand how additive substrates influence the biodegradation of aged PAHs in soils, more efforts should be devoted to investigating the abundance and community structure of culture-independent PAH-degraders during the process of PAH degradation.

In the present study, three widespread agriculture wastes including wheat stalk (WS), mushroom cultivation substrate waste (MCSW) and cow manure (CM) were selected to promote the microbial degradation of aged PAHs in soil microcosms. Three targeted PAH-degrading genes including *pdo1*, *C12O* and *nah*, which encode the pyrene dioxygenase, catechol 1,2-dioxygenase and naphthalene dioxygenase, respectively, have been frequently detected in aged PAHs-polluted soils (Johnsen et al., 2006; Han et al., 2014). The elimination efficiency of various PAHs and the abundance and community composition of the three PAH-degrading genes were monitored during the incubation of the aged PAH-polluted soil treated with various agricultural wastes. We tested the following hypotheses: (i) the dissipation of aged PAHs would be promoted by input of agricultural wastes, owing to the nutrition supply, but the elimination efficiency would differ across treatments amended with different agricultural wastes based on the variance of introduced nutrient content and microbial consortia; (2) Addition of agricultural wastes would influence elimination of soil PAHs through altering the abundance and community structure of the PAH-degrading microorganisms.

2. Materials and methods

2.1. Collection of agricultural wastes and physicochemical characterization

Agricultural wastes including WS, MCSW and CM were taken from a farm in Tai'an, Shandong province, China. All the samples were passed through a 1.0 mm mesh, and air-dried for seven days. Total carbon (TC), total nitrogen (TN) and total sulfur (TS) in these agricultural wastes were measured on an element analyzer (Vario EL III, Elementar, Hanau, Germany). Dissolved organic matter (DOM) was extracted from each of agricultural wastes by Milli-Q water at ratios of 1:20 (w/v, waste/water) for WS, 1:40 (w/v) for MCSW, and 1:20 (w/v) for CM, respectively. The mixtures were shaken at 200 rpm on a reciprocating shaker for 16 h, centrifuged at 12,000 rpm for 20 min at 4 °C and filtered through 0.45- μm polycarbonate membranes (Millipore, Ireland). Dissolved organic carbon (DOC) in DOM extracts was measured using a TOC analyzer (LiquiTOC, Elementar Co., Hanau, Germany). The detailed information about the basic properties of these agricultural wastes is shown in Table 1.

2.2. Soil collection and microcosm incubation

The aged PAH-polluted soils were collected from a coke factory with six years of production history in Xintai (35°54'N, 117°43'E), Shandong province, China. The collected soils were sieved through a 2-mm mesh, and divided into two portions. One portion was subject to measurement of basic physicochemical properties using conventional methods recommended by the Soil Science Society of China (Lu, 1999). TC, TN and TS of the soil samples were measured using an element analyzer (Vario EL III, Elementar). The detailed information about the soil basic characteristics is shown in Table 1. Another portion of soils was used to set up short-term laboratory microcosms in 480 ml plastic vials containing 100 g of soils (oven

Table 1
Basic properties of the PAH-contaminated soil and the three agricultural wastes.

	Soil	WS	MCSW	CM
pH	7.34	—	—	—
H ₂ O (%)	10.6	—	—	—
MWHC (%)	44.5	—	—	—
Sand (%)	48.3	—	—	—
Silt (%)	46.8	—	—	—
Clay (%)	4.88	—	—	—
TC (g kg ⁻¹)	38.8 ^d	367.2 ^a	274.0 ^c	281.0 ^b
TN (g kg ⁻¹)	1.63 ^d	8.49 ^c	17.9 ^b	25.2 ^a
TS (g kg ⁻¹)	1.65 ^d	6.06 ^b	9.05 ^a	4.91 ^c
C:N ratio	23.7 ^b	43.3 ^a	15.3 ^c	11.2 ^d
DOC (g kg ⁻¹)	0.15 ^d	18.2 ^b	29.2 ^a	13.7 ^c

Notes: WS, wheat stalk; MCSW, mushroom cultivation substrate waste; CM, cow manure; MWHC, maximum water holding capacity. Different letters following the means ($n = 3$) in the same row indicate significant differences.

dry-weight equivalent), and sealed with air-permeable caps. Four sets of treatments were established in triplicate: the control treatment without addition of agricultural wastes (CK), and the treatments added with 6.25 g of WS, MCSW or CM agricultural wastes. Soil moisture content was adjusted to 60% of maximum water holding capacity (MWHC) and maintained constant through periodic replenishment of water. Soil microcosms were kept at 28 °C in the dark for three months. Approximately 10 g of soils were sampled from each microcosm on days 5, 15, 30, 55 and 90 to determine the dynamics of 16 priority PAHs and microbial populations involved in PAH degradation.

2.3. Measurement of the PAHs contents

The 16 priority PAHs in soils were extracted using an ultrasonic method recommended by the USEPA (2000). The contents of 16 PAHs were determined on a GC-MS (HP7890-5975C, Agilent Technologies Inc., Wilmington, DE, USA) equipped with Agilent DB-5MS Column (30 m × 0.25 mm × 0.25 μm) with a flow rate of 1 ml min⁻¹ as previously described (Han et al., 2015).

A certified mixture containing 16 USEPA priority PAHs (AccuStandard, New Haven, CT, USA) and Hexamethylbenzene were used as the external and internal standards respectively (Han et al., 2015). PAHs were identified and quantified according to their retention time, target and qualifier ions, and a calibration curve was established with the ratio of external to internal standards. The recovery rate in the process of extraction and clean-up was determined using Phe-d10 spiked into the soils 24 h prior to extraction of PAHs.

2.4. Soil DNA extraction and quantitative PCR (qPCR) assays

The genomic DNA was extracted from 0.25 g of soil using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, USA) according to the manufacturer's instructions. The concentration and quality of the extracted DNA were measured with a ND-1000 UV–Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

The bacterial 16S rRNA gene and the three PAH-degrading genes *pdo1*, *nah* and *C120* were amplified on an iCycler iQ 5 thermocycler (Bio-Rad Laboratories, Hercules, CA, USA). The primer pairs, probe and PCR conditions are listed in Table S1. Standard curves for qPCR assays were constructed as previously described (Han et al., 2015).

2.5. Terminal-restriction fragment length polymorphism (T-RFLP) analysis

Our previous studies in the same coke factory showed that all of

the *pdo1* gene sequences in clone libraries were affiliated into *Mycobacterium* (Han et al., 2014), and thus only the *nah* and *C120* genes were analyzed with T-RFLP to determine the community structure of PAH degraders in the present study. The *nah* and *C120* genes were amplified with the fluorescently labelled primers using the same procedure as that used in the qPCR analysis. The PCR products of the *nah* and *C120* genes were purified with a Wizard SV gel and PCR Clean-up System (Promega). The *nah* gene PCR products were digested with the restriction endonucleases *RsaI* and *AluI* (TaKaRa) and the *C120* gene PCR products with *MspI* (Promega) according to manufacturer's instructions. The resultant terminal restriction fragments (TRFs) were resolved using a Genetic Analyzer (3130XL, Applied Biosystems, Foster City, CA, USA) and analyzed with the GeneMapper 3.7 software. TRFs less than 50 bp or with a percentage of less than 1% in the all T-RFs were discarded. The relative abundance of each TRF was calculated by dividing the peak area of the T-RF by that of total T-RFs.

2.6. Construction of clone libraries and phylogenetic analysis

The purified PCR products of the *nah* or *C120* genes from the initial soil samples were used to construct the clone libraries with the pGEM-T Easy Vector system (Promega) following the manufacturer's instructions. The positive clones from the *nah* and *C120* clone libraries were selected for sequencing. Clone sequences sharing a 97% identity for the *nah* gene or an 85% identity for the *C120* gene were classified into the same operational taxonomic unit (OTU) using the MOTHUR software (<http://www.mothur.org/>). The representative sequences of each OTU were subject to BLAST analysis against the GenBank database (<http://blast.ncbi.nlm.nih.gov/>) and used to construct the phylogenetic trees using the neighbour-joining method with 1000 bootstrap replicates in MEGA version 5.0 (Tamura et al., 2011). The representative sequences of the *nah* and *C120* gene clones used in phylogenetic trees have been deposited in the GenBank nucleotide sequence database, respectively.

2.7. Statistical analyses

The abundance of the bacterial 16S rRNA and PAH-degrading genes was log transformed prior to the statistical analysis. Two-way non-parametric analysis of variance (ANOVA) based on Scheirer-Ray-Hare test was used to assess the significance of the effects of different agricultural wastes, sampling time and their interactions on the abundance of targeted genes in SPSS 19.0. One-way non-parametric ANOVA based on Kruskal-Wallis test was implemented to determine the significant differences in the content of PAHs or the abundance of bacterial 16S rRNA and PAH-degrading genes across different treatments at the same incubation time. Non-metric multidimensional scaling (NMDS) analysis was performed to display the Bray-Curtis dissimilarity matrices based on the relative abundance of the *nah* and *C120* gene TRFs across different treatments. Additionally, a permutational multivariate analysis of variance (PERMANOVA) test was performed to compare whether the T-RFLP profiles of the *nah* and *C120* genes in different treatments were statistically different, by using the “adonis” function of the “vegan” package in R platform with 999 permutations. The difference at the level of $P < 0.05$ was accepted to be statistically significant.

3. Results

3.1. Properties of the soil and agricultural wastes

The soil is classified as neutral sandy loam according to the soil

taxonomy of US system. The contents of TC, TN, TS and DOC in agricultural wastes ranged from 274.0 to 367.2 g kg⁻¹, 8.49–25.2 g kg⁻¹, 4.91–9.05 mg kg⁻¹, and 13.7–29.2 g kg⁻¹, respectively, which were significantly higher than those in soil samples (Table 1). The contents of these chemical parameters were also significantly different across WS, MCSW and CM. The C:N ratio of the collected soil was 23.7 which was significantly lower than that of WS but higher than those of CM and MCSW.

3.2. Impacts of agricultural waste amendment on the degradation of PAHs

The recovery rates of standard Phe-d10 were from 78.3% to 97.6% and did not significantly change over the incubation time, indicating the validity of the extraction and purifying method. The 16 types of PAHs were divided into five groups based on the numbers of aromatic ring. The initial content of total PAHs in the soil was 36.1 mg kg⁻¹ dry soil, with a high proportion of 4-ring PAHs comprising 41.7% of total PAHs. The changes in the absolute contents of residual PAHs in soil microcosms at each sampling time are illustrated in Fig. 1. The soils were taken from the sites with six years of coking production history, and therefore the PAHs in the soils had been considered as the aged ones at the start of microcosm establishment. Here, aging of PAHs over the short incubation time was neglected, and the differences of residual PAHs were only analyzed in different treatments at the same incubation time. No significant decline in the PAHs content was observed for all the treatments compared with CK at the first three sampling points. However, a significant positive effect of agricultural wastes on the degradation of PAHs was observed in the later period of incubation. There were significantly less residual PAHs in MCSW and CM on day 55 compared with CK. At the end of incubation, 24.4% of total PAHs relative to their initial content in the collected soils were eliminated in CK, while a greater removal rate of 33.3–40.4% was achieved in treatments added with agricultural wastes (Table 2). Furthermore, the PAHs with different numbers of aromatic rings differed in their

degradation rates. The PAHs with low molecular weight (LMW) had higher degradation rates of 40.7–61.2%, compared to 18.7–33.1% for those with high molecular weight (HMW) in all soil microcosms. However, dissipation of the 5-ring PAHs was faster than the 4-ring PAHs when added with agricultural wastes.

3.3. Impacts of agricultural waste amendment on the abundances of the 16S rRNA, *pdo1*, *C120* and *nah* genes

The initial absolute abundances of the 16S rRNA, *pdo1*, *C120* and *nah* genes in soil microcosms were 4.66×10^{10} , 3.13×10^9 , 5.06×10^6 and 2.10×10^6 copies g⁻¹ dry soil, respectively. The log transformed copy numbers of these genes during the incubation period are illustrated in Fig. 2. The non-parametric two-way ANOVA analysis showed that the treatments with agricultural wastes exerted significant influence on the abundance of all targeted genes. The incubation time and its interaction with treatment had also significant effects on the abundances of some detected genes (Table S2).

The non-parametric one-way ANOVA was further implemented to determine the effects of different agricultural wastes on the abundance of the targeted genes at the same incubation time. Overall, the added agricultural wastes had significant and positive effects on the abundance of some targeted genes, but these genes showed different patterns responding to the amendment of agricultural wastes. The bacterial 16S rRNA gene abundance was significantly higher in CM and MCSW treatments than that in CK during most of the incubation period. The *pdo1* gene was more abundant in CM and MCSW treatments than CK after 5 days of incubation. The *C120* gene abundance did not significantly increase in all treatments during the whole incubation period. Addition of MCSW greatly enhanced the *nah* gene abundance compared to CK on days 15, 55 and 90 of incubation (Fig. 2).

3.4. Community structure of the *nah* and *C120* genes in soil microcosms

The T-RFLP analysis of the *nah* gene yielded four distinct TRFs, of which TRF-325 was the most dominant phylotype with a proportion ranging from 55.8 to 80.4% (Fig. 3a). In general, amendment of the three agricultural wastes obviously increased the relative abundance of TRF-325 but decreased the TRF-221 abundance during the early and middle stages of incubation. Similarly, the relative abundance of TRF-260 in WS and MCSW was observed to increase in the early period of incubation. No significant effects of agricultural wastes on the patterns of TRF-92 could be observed. The PERMANOVA analysis showed that T-RFLP profiles of the *nah* gene were significantly different across the different treatments (Pseudo- $F = 24.7$, $P < 0.001$). The NMDS ordination showed that the

Fig. 1. Changes in the absolute contents of PAHs in soil microcosms at different incubation periods. CK, WS, MCSW and CM represent the control and the treatments with wheat stalk, mushroom cultivation substrate waste and cow manure, respectively. -5, -15, -30, -55 and -90 mean the incubation periods of 5, 15, 30, 55 and 90 days, respectively. Error bars indicate standard deviations ($n = 3$). Different letters in the same column indicate significant differences of the total PAH contents across different treatments at the same incubation time.

Table 2

The proportions (%) of soil PAHs with different rings degraded at the end of incubation relative to their initial content in soils collected from the coke factory.

	CK	WS	MCSW	CM
2-ring PAHs	49.2	74.3	62.3	64.0
3-ring PAHs	36.1	51.2	55.8	59.7
4-ring PAHs	19.8	24.9	31.6	32.7
5-ring PAHs	18.4	26.5	38.0	39.2
6-ring PAHs	15.8	18.8	19.2	24.9
LMW PAHs	40.7	59.3	60.2	61.2
HMW PAHs	18.7	24.3	31.2	33.1
total PAHs	24.4	33.3	38.7	40.4

CK, WS, MCSW and CM represent the control and the treatments with wheat stalk, mushroom cultivation substrate waste and cow manure, respectively. Data shown represent the mean values of three replicates.

Fig. 2. Changes in the absolute abundances of the bacterial 16S rRNA (a), *pdo1* (b), *C120* (c) and *nah* (d) genes in soil microcosms at different incubation periods. CK, WS, MCSW and CM represent the control and the treatments with wheat stalk, mushroom cultivation substrate waste and cow manure, respectively. Error bars indicate standard deviations ($n = 3$).

treatments with agricultural wastes had a clear divergence from CK, and the CM treatment was also obviously separated from other treatments (Fig. 4a).

A total of 10 TRFs were obtained for the *C120* gene (Fig. 3b). TRF-139 was the most prevalent phylotype with a proportion ranging from 11.9 to 83.7%. TRF-122, TRF-157, TRF-86, TRF-83 and TRF-210 were also abundant phylotypes. The proportion of TRF-139 markedly increased, while TRF-86 and TRF-122 obviously declined in CM compared to CK. The percentages of TRF-157, TRF-210 and TRF-83 showed a rising trend in WS and MCSW. The PERMANOVA analysis showed that T-RFLP patterns of the *C120* gene were significantly distinct across different treatments (Pseudo- $F = 25.2$, $P < 0.001$). The NMDS analysis showed a divergence of treatments with agricultural wastes from CK (Fig. 4b). In particular, the T-RFLP profile of the *C120* gene in CM obviously separated from other treatments throughout the whole incubation time.

3.5. Phylogeny of the *nah* and *C120* genes in soil microcosms

A total of 54 sequences from the *nah* gene clone library were assigned into 11 OTUs. The representative sequences from each OTU, which were used to construct the phylogenetic tree, contained all TRFs types. All clones of the *nah* gene were affiliated within *Betaproteobacteria* (59.3%) and *Gammaproteobacteria* (40.7%) (Fig. 5a). The clones of TRF-325 and TRF-92 were classified into *Betaproteobacteria*, while those of TRF-260 and TRF-221 were clustered into *Gammaproteobacteria*.

A total of 143 sequences from the *C120* gene clone library were assigned into 19 OTUs, and covered all TRFs types. The phylogenetic tree showed that all of the *C120* clones were grouped into *Gammaproteobacteria* (66.4%) and *Betaproteobacteria* (33.6%) (Fig. 5b). For the dominant TRF-139, 86 TRFs were found in *Gammaproteobacteria* and two TRFs in *Betaproteobacteria*. TRF-210 only emerged

in *Gammaproteobacteria*, while the TRF-122, -92, -157, -194, -97 and -110 existed in *Betaproteobacteria*. Five of seven TRF-86 clones were classified into *Gammaproteobacteria*.

4. Discussion

4.1. Impacts of agricultural wastes on the degradation rates of PAHs

Biostimulation is one of the most widely used microbial strategies to remove PAHs from soils (Sayara et al., 2011). In this study, we found that addition of three common agricultural wastes enhanced the dissipation of PAHs in soil microcosms, but they had different removal efficiencies of total PAHs. Although carbon source was considered as a major nutrient for bacterial growth, the C:N ratio was also suggested to be an important factor affecting the growth of soil microorganisms (Zhou and Crawford, 1995). Previous studies have demonstrated that the optimum ratio of soil C:N for microbial growth was around 10 (Teng et al., 2010). In this study, the C:N ratio of the soil was 23.7, and addition of WS with a high C:N ratio (43.3) would further elevate the C:N ratios in the soil microcosms. By contrast, the introduced CM (with a C:N ratio of 11.2) and MCSW (with a C:N ratio of 15.3) would strongly reduce the soil C:N ratio, and it might partially explain the observation that addition of CM and MCSW significantly accelerated the dissipation of soil PAHs on day 55 compared to the control. Therefore, we supposed that an appropriate C:N ratio might play a crucial role in determining the effects of agricultural wastes on the microbial degradation of PAHs especially under nitrogen-depleted conditions. On the other hand, input of carbon-rich materials could promote the adsorption of soil PAHs, which was assumed to be an important factor impeding microbial bioremediation of the aged PAHs in soil (Kobayashi et al., 2009). On the contrary, nutrient sources enriched in nitrogen have been known to enhance the

Fig. 3. T-RFLP patterns of the *nah* (a) and *C120* (b) genes across different treatments in soil microcosms at different incubation periods. CK, WS, MCSW and CM represent the control and the treatments with wheat stalk, mushroom cultivation substrate waste and cow manure, respectively. –5, –15, –30, –55 and –90 mean the incubation periods of 5, 15, 30, 55 and 90 days. Error bars indicate standard deviations ($n = 3$).

bioavailability of aged PAHs (Teng et al., 2010; Pelaez et al., 2013). Therefore, these findings together with our study suggested that substrates with appropriate C:N ratios could facilitate the remediation of soils contaminated by aged PAHs. Except for C:N ratio, microbial degraders may be affected by introduced metals and trace elements, thus effects of the diverse constituents on PAH dissipation should be fully considered when selecting feasible biostimulation materials.

In this study, we observed that HMW PAHs were degraded more slowly than LMW PAHs (Table 2). It is well known that the aqueous solubility and bioavailability of PAHs declines with the increasing numbers of aromatic rings of PAHs. As a consequence, HMW PAHs were more resistant to microbial degradation than the LMW PAHs (Peng et al., 2008). In the control, the dissipation of PAHs with different aromatic rings was completely in accordance with the degradation rule. However, the 5-ring PAHs were dissipated faster than the 4-ring PAHs in the treatments with agricultural wastes, which is in line with previous studies (Potin et al., 2004; Li et al., 2012). It was speculated that the faster dissipation of 5-ring PAHs than 4-ring PAHs might be attributed to the added agricultural wastes. Although PAHs with above five aromatic rings were highly recalcitrant under natural conditions, they could be biodegraded efficiently where co-metabolic substrate existed after addition of agricultural wastes (Johnsen et al., 2005; Haritash and Kaushik, 2009). In addition, some studies have demonstrated that *Mycobacterium* had a good catabolic capacity towards degrading PAHs up to five benzene rings due to their lipophilic surfaces and biosurfactant production (McClellan et al., 2002; Bogan et al., 2003). Therefore, the

Fig. 4. NMDS ordinations based on the Bray-Curtis similarity indices of the T-RFLP data of the *nah* (a) and *C120* (b) genes across different treatments. CK, WS, MCSW and CM represent the control and the treatments with wheat stalk, mushroom cultivation substrate waste and cow manure, respectively. –5, –15, –30, –55 and –90 mean the incubation periods of 5, 15, 30, 55 and 90 days.

abundant and ever-increasing *Mycobacterium* harboring the *pdo1* gene during the incubation might play an important role in eliminating 5-ring PAHs in the treatments with agricultural wastes.

4.2. Impacts of agricultural wastes on the abundances of PAH-degrading genes

Irrespective of the nature and chemical structure of the different types of PAHs, the degradation of PAHs is assumed to be mainly dependent on the abundance and composition of microbial populations with the capacity of degrading PAHs (Haritash and Kaushik, 2009). In this study, the increased abundance of the bacterial 16S rRNA and the PAH-degrading genes (*pdo1* and *nah*) was in coincidence with the faster attenuation of PAHs in soil microcosms treated with agricultural wastes relative to the control, which supported the assumption that agricultural wastes could improve the biodegradation of PAHs through increasing the abundance of PAH-degrading populations in soils. Previous studies have identified some important PAH-degrading microorganisms such as *Mycobacterium* harboring the *pdo1* gene and *Betaproteobacteria* and *Gammaproteobacteria* carrying the *nah* gene (Johnsen et al., 2006; Han et al., 2015). Our results found that addition of agricultural wastes could impose positive effects on the abundances of these genes. It might be attributed to the nutritional components (e.g. carbon, nitrogen, and phosphorous etc.) constantly provided for soil microbes by the slow-releasing agricultural wastes (Teng et al., 2010; Pelaez et al., 2013). Several previous studies also demonstrated that supply of carbon, nitrogen and phosphorous sources could enhance the proliferation of soil microorganisms and hydrocarbon degraders (Vinas et al., 2005; Teng et al., 2010; Pelaez

Fig. 5. Phylogenetic trees of the *nah* (a) and *C120* (b) gene sequences retrieved from the soils contaminated by aged PAHs. Designation of the clones included the following information: clone code, followed by the accession number in the GenBank, T-RF length and number of the clone with above 97% (for *nah*) and 85% (for *C120*) similarity to the listed representative sequence in the parentheses. Bootstrap values (>50%) were indicated on nodes. The scale bars represent 2% (a) and 5% (b) estimated sequence divergences.

et al., 2013). Aside from the biostimulation caused by nutrient input, agricultural wastes contain microbial populations, and thus might also have an effect of bioaugmentation on soil microbial populations (Li et al., 2012).

4.3. Impacts of agricultural wastes on the community structure of PAH-degraders

In this study, all the *nah* and *C120* clones were affiliated within *Betaproteobacteria* and *Gammaproteobacteria*, which indicated that these two bacterial taxa are potential important PAH degraders in soils (Lors et al., 2010; Niepceon et al., 2013). It was observed that the CM treatment exerted a remarkable influence on the T-RFLP patterns of PAH degraders. As for the T-RFLP profile of the *nah* gene, some TRFs such as TRF-260 affiliated into *Gammaproteobacteria* were apparently different from the control. The T-RFLP pattern of the *C120* gene also obviously showed that the CM treatment was always different from other treatments. In addition, various agricultural wastes imposed discrepant influences on the T-RFLP profiles of the *nah* and *C120* genes. The CM treatment seemed to have a more persistent effect on community structures of the *nah* and *C120* genes while those in WS and MCSW had a trend of recovery towards the control in the later incubation time. It indicated that addition of agricultural wastes had a significant effect on community components of the *nah* and *C120* genes, and various agricultural wastes usually imposed different influences on the community structure of the PAH-degrading genes (Han et al., 2015). Li et al. (2012) found similar results that the community structure of bacteria and fungi showed different responses to addition of mushroom cultivation substrate in PAH-contaminated soils. Overall, the abundances of the PAH-degrading genes were not significantly higher in CM than those in other treatments in this study. However, the PAHs in CM were eliminated more quickly than other treatments. It was probably due to the notable changes in the community structure of PAH degraders in CM. In contrast to WS with high content of refractory hemicellulose, cellulose and lignin

(Zhang et al., 2016) and MCSW with probably poor fungal colonization (Li et al., 2012), CM might harbor more abundant active microorganisms (Girija et al., 2013) and had a significant influence on microbial consortia in soil. In summary, although various agricultural wastes affected PAH-degrading genes in different ways, our results indicated that external stimuli produced by agricultural wastes enhance the microbial degradation of PAHs in soils through not only enhancing the abundance but also altering the community composition of microbial PAH degraders.

5. Conclusions

This study provided evidence for the modulation of agricultural wastes on dissipation of aged PAHs most likely through influencing the abundance and community structure of soil microbial degraders. We found that all treatments amended with agricultural wastes enhanced the elimination of soil PAHs, and the agricultural wastes with low ratios of C:N could more effectively enhance the attenuation of PAHs than that with relatively high ratios of C:N. The addition of agricultural wastes significantly improved abundances of the PAH-degrading genes (i.e. *pdo1* and *nah*) and bacterial 16S rRNA gene, and changed the community structure of the *nah* and *C120* genes. Our findings contribute to the understanding of how the bioremediation with agricultural wastes regulate the depletion of aged PAHs from polluted soil environments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.chemosphere.2017.01.012>.

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