

## Comparison of Phytoremediation on Polycyclic Aromatic Hydrocarbons in Sludge between Awnless Brome and Alfalfa and its Potential Mechanism

Chang Rui-xue<sup>1,3</sup>, Feng Sheng-dong<sup>1\*</sup>, Yang Zhi-xin<sup>1\*</sup>,  
Li Yu-ling<sup>2</sup> and Zhao Ou-ya<sup>1</sup>

<sup>1</sup>Key Laboratory for Farmland Eco-Environment, Hebei Province and College of Resource and Environmental Sciences, Agricultural University of Hebei, Baoding - 0710001, P.R.China.

<sup>2</sup>College of Forestry; Agricultural University of Hebei

<sup>3</sup>College of Resources and Environmental Sciences, China Agricultural University, Beijing 100193, P.R.China.

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Polycyclic aromatic hydrocarbons (PAHs) in urban sewage sludge are becoming serious environmental pollution and phytoremediation is a new technology for the remediation of soil organic pollutants. The purpose of current study was to compare the phytoremediation effect on PAHs between Awnless Brome and Alfalfa and to explore the potential mechanism of the phytoremediation effect. By pot experiment of Awnless Brome and Alfalfa in greenhouse, we compared the repaired effect of PAHs in the sludge, soil enzyme activities near plant rhizosphere area and bacterial species diversity. The results showed that PAHs in the sludge can be restored by the two plants directly. Awnless Brome had a 7% higher removal rate for PAHs than Alfalfa in total. The range of removal rates of single PAHs was about 24.69%~97.69% for Awnless Brome, and 29.69%~97.72% for Alfalfa. As compared with Alfalfa, Awnless Brome had a removal rate of 53%, 13% and 9% higher for PAHs with 2, 3 and 4 rings, 5% and 11% lower for PAHs with 5 and 6 rings, respectively. We also found that the soil enzymes activities and microbial diversity were related with the removal rate of PAHs. The activities of catalase and polyphenol oxidase near Alfalfa root zone were significantly higher than Awnless Brome root zone in sludge, which was opposite for dehydrogenase. Using the PCR-DGGE method, we found one common microbe band related PAHs degradation in rhizospheric area of Awnless Brome and Alfalfa, two specific microbe bands for Alfalfa, and six specific microbe bands for Awnless Brome. In conclusion, the two plants, Awnless Brome and Alfalfa, would be directly used for remediation of PAHs-polluted sludge.

**Key words:** Sewage Sludge, PAHs, Awnless Brome, Alfalfa, Enzyme Activities, Microbes.

The determination of hazardous substances in sewage sludge is becoming an important issue due to increased amounts of sewage sludge produced as a consequence of the rising number of wastewater treatment plant. The sludge in agriculture could be advantageous for their organic matter and nutrients. But, utilization as a fertilizer has some risks also, such as the accumulation of pollutants in soils, plants, animal

pastures and the subsequent entry into the food chain of its dangerous compounds<sup>1-3</sup>; PAHs is one of the persist organic pollutants (POPs) in sludge, especially because of their omnipresent presence and persistence in air, water, and soil, as well as their toxicity to humans and other living organisms<sup>4</sup>. Though PAHs may be fading away from the environment through adsorption, chemical degradation, photolysis, and volatilization, microbial degradation is still the major process to remove PAHs contamination from the environment<sup>5</sup>.

\* To whom all correspondence should be addressed.  
Yang Zhi-xin, PhD, E-mail: yangzhixin@126.com  
Feng Sheng-dong, E-mail: fengshengdong@126.com

Phytoremediation, as a new field of environmental remediation, is referred to the use of plants and their associated microorganisms for restoring and/or recovering polluted soils<sup>6</sup>. Phytoremediation is likely to be effective in the disappearance of pollutants from planted soils<sup>7-11</sup>, whereas its efficacy varies greatly among plant species<sup>12,13</sup>, soil and environmental conditions<sup>14</sup>, and the physicochemical nature of the contaminant<sup>15,16</sup>. Forage grasses are getting more and more attentions because they have remarkable effect on ameliorating the physicochemical properties by reestablishing initial pH values ( $>7.0$ ) and significantly reducing salinity ( $EC < 500 \mu S \text{ cm}^{-1}$ ), especially alfalfa (*Medicago sativa* L.) and ryegrass<sup>17-22</sup>. Phytoremediation used for PAHs in soil have been reported a lot<sup>23-25</sup>, but little is found for sludge. The overall objective of this study was to compare the ability of degrading PAHs in the sludge between Awnless Brome and Alfalfa. The relationships between the degradation and enzyme activity, as well as between the degradation and microbial activity, were also investigated in order to explore the potential mechanism of phytoremediation effect on PAHs.

## MATERIALS AND METHODS

Sludge was taken from domestic wastewater treatment plant in Baoding city, Hebei province of China. After the natural drying in the shade and sieving of the samples, set aside. The seeds of Awnless Brome and Alfalfa were purchased from Jingjingyuanyi seed company, which were Chinese hybrids. Basic components of sludge included 23.47g/kg TN; 0.6993g/kg available P and 3.8634g/kg available K, while total PAHs concentration was 1822.97  $\mu\text{g/kg}$ , includ 16 PAHs as followed : Naph, Acy, Ace, Flu, Phe, Anth, Flt, Pyr, BaA, Chry, BbF, BkF, BaP, DbA, BghiP and InP.

### Experimental design

Pot experiment was set in greenhouse of Agriculture University of Hebei with pots of 15×20cm (diameter×height). There were two treatments with one plant species, each treatment had three repetitions. The plants were two kinds of forage grass, Awnless Brome and Alfalfa. The seeds were soaked for 16 hours, put on a petri dish. The seeds were put in two piece of filter paper

soaked enough water. Put the petri dish in a constant temperature incubator for sprouting, then the seeds were moved to the pot. Thinned out the sprouts after 15 days, left 10 sprouts every pot. Grown for 5 months, the plants were reaped.

Moisture in the pot were maintained at 60% of field capacity, the temperature of the greenhouse were 20 to 23 °C in the day and 17 ~ 18°C in the night. Light intensity of 4500 ~ 7300 lx. The positions of the basins were randomly changed every 2 days in the greenhouse.

### Sampling and analysis of plants and sludges

Sludge samples collection in rhizospheric area: The sludge samples were gathered after plants harvest, while the range was 1mm inside the roots , keep in 0-4° areas and set aside for PAHs , enzyme activity and microbial activity analysis.

Plant samples: Plants was harvested after 5 months growth , washed and smashed , keep in 0-4°C areas and set aside for PAHs analysis.

### PAHs analysis of plants and sludges

1. Samples extraction, Samples (20 g) of dried sludge were extracted for 16h with dichloromethane (200 ml) in a Soxhlet apparatus. Silica gel column was clean up and used for the purification of the extract according to EPA method 3630 (EPA, 1994). The extracts were concentrated to 0.5 ml under nitrogen at 60°C and analyzed by GC-MS. Deuterated PAHs (Naphthalene-d8, Ace-naphthene-d10, Phenanthrene-d10, Chrysene-d12 and Perylene-d12) were used as internal standards and were added to the sludge prior to extraction.
2. Choice of GC-MS conditions for PAH analysis: GC-MS analyses were carried out using a Varian Model 3400 Cx gas chromatograph connected to a Varian Saturn 3 (Ion Trap) mass selective detector, interfaced with a Saturn GC-MS data system (revision 4.0) for data acquisition, processing, and quantification.<sup>27</sup>

The chloroform spectrometer operating conditions: the sample injector temperature was set to 280°C, not separated while once 2 $\mu\text{L}$ ; column flow was 1.1ml/min, constantly; the temperature was programmed from 70°C, held for 4min, from 70°C to 300°C at 10°C/min, held for 2min, followed by an increase to 340°C at 5°C/min and held to the end.

The mass spectrometer operating conditions: the Ion source temperature was set to 230°C, while quadrupole was 150°C; acquisition in scan-mode with a range of  $m/z$  40~ $m/z$  500; choosing Quantitative analysis for ions<sup>26</sup>. The way of ion detection was Selected-Ion-Monitoring (SIM).

Quality control: recovery rate and detection line were refer to the EPA standard method.

#### Sludge Enzyme Activities Analysis in the rhizosphere

Enzyme activities were determined on fresh moist sludge sieved <2 mm.

Catalase (CAT) activity was measured with the standard method, potassium permanganate titration<sup>28</sup>. One unit of catalase activity decomposed 1.0  $\mu$ mole H<sub>2</sub>O<sub>2</sub>/min (pH 7.0, 25°C). Urease (UR) activity was measured as described by Kandeler and Gerber<sup>29</sup>; the activity was expressed as micrograms of substrate hydrolysed at 30°C h<sup>-1</sup> by 1 g of dried sludge.

A unit (U) of Dehydrogenase (DH) assays was performed using soluble tetrazolium salt (TTC) as an artificial acceptor<sup>30</sup>; the activity was defined as the micromoles of substrate transformed at 30°C h<sup>-1</sup> by 1 g of dried sludge.

The polyphenol oxidase (PPO) activity was measured by the method of Kunwar and Khan<sup>31</sup>. The amount of purpurogallin formed was estimated by measuring the absorbance at 420 nm; the activity was expressed per minute per gram fresh weight.

#### Diversity Analysis of Sludge Microbial Flora (Bacteria) in the rhizosphere

1. DNA Extraction: M-NDA Marker was from Tiangenshenghua Technology Co. in Beijing 1kb plus DNA Ladder(MD113) used Ultra Clean sludge DNA Kit (MoBio Laboratories, Inc., So lina Beach, California).
2. PCR Amplification: the 16S rDNA was performed on the extracted DNA, by using eubacterial universal primers P27f and P1495r referred to *E.coli* nucleotide sequence of 16S rDNA gene<sup>32</sup>. Nested PCR reaction for V3 amplification was carried out according to Muyzer and Smalla<sup>33</sup>.
3. DGGE Electrophoresis: V3 PCR products from sludge, enrichment culture and bacterial isolates DNAs were characterized

by a DGGE run on a vertical acrylamide gel in a DCODE Universal Mutation Detection System (Biorad). DGGE was performed with 8%(wt/vol) polyacrylamide gels in TAE buffer (20 mM Tris acetate pH 7.5, 10 mM sodium acetate, 0.5 mM Na<sub>2</sub>-EDTA) with a linear chemical gradient ranging from 35% to 65%. Gels were run at a constant voltage of 150 V for 7 h. The DNA bands were visualized by silver staining coupled with digital scanning. The silver staining procedure was performed as described by Bassam *et al.*<sup>34</sup>. To get a clear image, the gel was photographed with gel photo system<sup>35</sup>.

#### Data analysis

##### Removal Rate of PAHs in Sludge

Removal Rate=(initial concentration-concentration after 5months) / initial concentration\*100%

##### Accumulation Contribution Rate of overground pats of plants

Accumulation Contribution Rate=Amount of PAHs in plants /removal amount of PAHs\*100%

(3) Shannon-Weaver index of diversity(H)<sup>36</sup>  $H=-\sum P_i \ln P_i$

(4) Shannon equitability index(E)<sup>37</sup>  $E=H/\ln S$

(5) Simpson dominance index(D)<sup>39</sup>  $D=1-\sum P_i^2$

where pi was the relative surface intensity of each DGGE band, S was the number of DGGE bands (used to indicate the number of species) and N was the sum of all the surfaces for all bands in a given sample (used as estimates of species abundance)<sup>38</sup>.

Data in this paper was analyzed with Excel and SPSS17.0. Contiguous sequences from the same PCR product were edited in the MEGA3.1 software package<sup>40</sup> and assembled using the Clustal Walgorithm<sup>41</sup> in MEGA3.1. In the microbial flora analysis, cluster analysis was made with MEGA3.1 after a series of file conversion, while principal component analysis was made with SPSS17.0 after a series of file conversion as well.

## RESULTS AND DISCUSSION

#### Removal Rates with Awnless Brome and Alfalfa

The average removal rates of total PAHs and single PAH associated with Awnless Brome and Alfalfa were shown in Fig.1. The removal rate

of total 16 PAHs was 84.3% for Awnless Brome and 77.2% for Alfalfa. Awnless Brome was 7% higher than Alfalfa. Compared the removal rates of single PAHs between the two plants, the removal rates for Naph, Acy, Ace, Flu, Phe, Flt, Pyr, BaA, Chry, BbF and BkF were higher Awnless Brome than by Alfalfa; In detail, more than 80% of Naph, Phe, Flt, Pyr, BaA, Chry, BbF, BkF and InP, and more than 90% of BaA and BkF were removed by Awnless Brome. Removal rates for single PAHs were in the range of 24.69%~93.46% by Awnless Brome. In contrast, removal rates for Anth, BaP, DbA, BghiP and InP were higher with Alfalfa, the range of removal rates was 29.69%~97.72%, removal rates of single PAHs increased with the increase of PAHs rings. BaA, Chry, BkF, DbA and InP were removed more than 80% by planting Alfalfa, especially for DbA and InP, the removal rates were more than 95%. So we could say that BaA, Chry, BkF and InP can be efficiently removed by either treatment. When we classified the PAHs by rings, as shown in Fig. 2, removal rates by Awnless Brome was ruleless between PAHs-rings and average removal rates for PAHs with different rings, while with Alfalfa the relationship can be described by logarithmic equation ( $R^2 = 0.910$ ).

Awnless Brome had a 53% higher removal rates for PAHs with 2 rings than Alfalfa, while the removal rates for PAHs with 3-4 rings was 13% and 9% higher for than Alfalfa. However, Alfalfa had 5% and 11% higher removal rates for PAHs with 5 and 6 rings than Awnless Brome. Lee indicated that 99% of phenanthrene and 77~94% of pyrene could be degraded in planted soil where the PAHs were added<sup>42</sup>. While Song had reported

a relatively lower removal rates when soil was polluted for a long time<sup>44</sup>, PAHs in soil became more stable after a long time so that the degraded effect would be decreased<sup>46</sup>. At the same time, microbial activities and enzyme activities should be higher in sludge than in soil<sup>47</sup>. Furthermore, in the experiment of early study, Alfalfa had much better effect than Awnless Brome for PAHs degradation of farmland soil of Wastewater Irrigation<sup>44</sup>, which was opposite in sludge in this study. The reason may be the PAHs utilizability in sludge was more suitable for Awnless Brome than Alfalfa, and Awnless Brome could be studied for sludge usage in the future.

As PAHs may undergo adsorption, volatilization, photolysis, and chemical degradation, we also calculated the plants accumulation as shown in Table 1 (Just the PAHs that accumulation contribution percent over 0.1% were put in the table 1). In total, Awnless Brome and Alfalfa had an accumulation percent of 0.83% and 0.19%, respectively. For the 16 PAHs, only 9 of them had accumulation contribution rates more than 0.1%. Among them, Alfalfa had a 0.19% higher accumulation contribution rate just for Naph, as compared with Awnless Brome. The findings implied that Awnless Brome could absorb more PAHs from sludge, and the kinds that can be absorbed were 2-4 rings PAHs rather than PAHs with 5-6 rings, except DbA which can be absorbed more than 0.1% in pots planting Awnless Brome. The amount of Phe absorbed by alfalfa and Awnless Brome from sludge was the highest among all the PAHs, up to 0.69% and 3.33% respectively. Our finding indicated that Awnless Brome was useful

**Table 1.** Contributions of Plants Accumulation (%)

Type	Naph	Acl	Ace	Flu	Phe	Anth	Flt	Pyr	DbA	sum
Awnless Brome	0.24	1.03	1.39	2.08	3.33	2.73	1.59	0.73	0.49	0.83
Alfalfa	0.43	0.03	0.53	0.42	0.69	0.46	0.50	0.49	0.01	0.19

**Table 2.** Enzyme Activity of Rhizospheric Sludge

Type	Catalase (CAT)	Urease(UR)	Dehydrogenase (DH)	Polyphenol Oxidase(PPO)
Awnless Brome	4.7895a	2.0316a	22.1293b	1618.1830a
Alfalfa	5.1596b	2.0402a	12.1497a	2800.8408b

Values followed by the same letter are not significantly different ( $p < 0.05$ , by SPSS).



Fig.1. The Removal Rates of Single and Sum of 16 PAHs

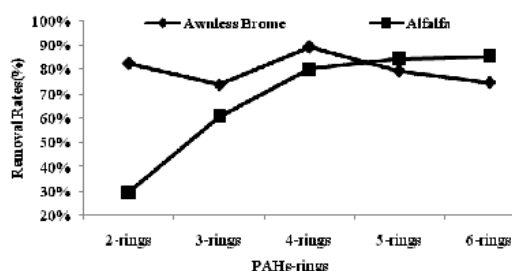


Fig. 2. The Removal Rates of PAHs with n-rings

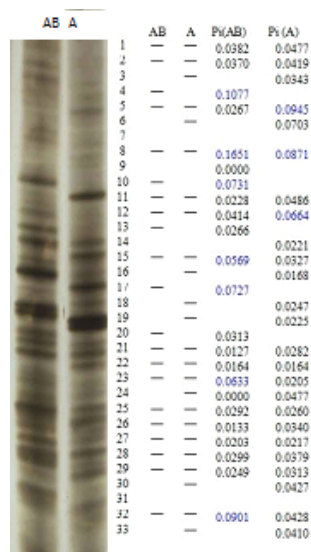


Fig. 3. PCR-DGGE picture and Pi of microbes in Rhizosphere area of Awnless Brome(AB) and Alfalfa(A)

Table 3. Microbial Diversity Index

Type	S	H	D	E
Awnless Brome	21	2.8007	0.9349	0.9243
Alfalfa	25	3.1039	0.9643	0.9498

for PAHs absorption from sludge, especially for PAHs in low molecular weight, which may be resulted from the activity of enzyme or microbial as presented in the next session.

#### Relationship between Removal Rates and Sludge Enzyme Activity

The activities of the four enzymes of the investigated sludges were reported in Table 2. Significant differences were observed between Awnless Brome and Alfalfa in the activities of Dehydrogenase (DH) and Polyphenol Oxidase (PPO). The activities of PPO in Alfalfa's rhizospheric sludge were significantly higher than those in Awnless Brome, while was opposite for DH.

Considering the removal characteristics by planting Awnless Brome and Alfalfa, it may be caused by different activities of enzymes in rhizospheric area. The removal rates of Awnless Brome for PAHs with 3-4 rings were 13% and 9% higher than Alfalfa. Awnless Brome had a 53% higher removal rates for PAHs with 2 rings than Alfalfa, which may be related with DH, for the activity was significantly higher in Awnless Brome than Alfalfa, and DH typically occurs in all intact, viable microbial cells, and was usually related to the presence of viable microorganisms and their oxidative capability<sup>43</sup>, it showed that the enzyme may be generated by a microbe which could help degraded the PAHs with 2-4 rings and DH activity may be related with PAHs absorption by Awnless Brome. Alfalfa had 5% and 11% higher removal rates for PAHs with 5 and 6 rings than Awnless Brome, the difference were not so significant as removal rates for PAHs with 2 rings. PPO is an important oxidoreductase in soils and can catalyse the degradation and transformation processes of aromatic compounds<sup>45</sup>, which could illustrate that the removal rates for PAHs with 5-6 rings were higher



by Alfalfa than Awnless Brome, and PPO were related with high molecular weight PAHs degradation.

The activities of CAT and UR was not significantly different between two kinds of plant, the difference between the two enzymes in rhizospheric areas of Awnless Brome or Alfalfa were greatly lower than that of the other two enzymes. The relationship between removal rates and the two enzymes activity was not so significant, and few references can be found to explain the significant relations, it need to do more deep research in the next step.

#### **Relationship between Removal Rates and Microbial Activities in Sludge**

In our analysis, the number of DGGE bands was treated as an indication of species in each sample. Bands(S), H, E and D were put in table 3 below. All of them were greater in the rhizospheric area of Alfalfa, which suggested that Alfalfa could give more contribution for microbial population and microbial activity totally. This phenomenon was consistent with the characteristic of enzymes activity as showed above, which illustrated that planting Alfalfa could increase the activities of enzyme and microbes in rhizospheric area. This may be characteristic of Legume, for they has their own Rhizobium so that can fix nitrogen by themselves<sup>48</sup>. But considering the removal rates and the activities of enzymes and microbes, we concluded that even the plants can really help degrade PAHs, not all activities of enzymes and microbes meant high effect for PAHs degradation. Overall, certain kind microbe for degradation should be identified.

DGGE profiles of sludges and Pi of all bands were shown in Fig. 3. Many DGGE bands were observed in the profiles, which indicating the presence of different bacterial populations and different relative abundance species in the sludge. Pi indicated that which one should be a dominant population. There were 6 kinds of microbes whose Pi was more than 0.05 in rhizospheric area of Awnless Brome while just 3 microbes was found for Alfalfa. These bands may be in relationship with PAHs among the degradation in the rhizospheric area of sludge with PAHs. Among the 6 bands, band 8 were dominant population both in rhizospheric area of Awnless Brome and Alfalfa which may be the microbes which can degrade PAHs. For the high efficiency of degradation by

Awnless Brome and Alfalfa, we can conclude that band 8 may represent a microbe effective for degradation of BaA, Chry, BkF and InP. Band 5 and band 12 were dominant population just in rhizospheric area of Alfalfa which may tell a relation between PAHs with 5-6 rings and the two microbes. Other bands whose Pi were more than 0.05, except band 8, in rhizospheric of Awnless Brome may be effective for 2-4 rings PAHs degradation. DNA sequence was warranted in future studies in this research field to find kinds of these microbes, which maybe helpful to identify certain kind of microbes and help to find a PAHs-degrading bacteria in sludge distinguished with which in soil.

#### **CONCLUSION**

In conclusion, the two plants tested in this study, Awnless Brome and Alfalfa, were all capable of removing PAHs in sludge. Awnless Brome had a 7% higher removal rate for total PAHs than Alfalfa, while Alfalfa could remove more PAHs with 5 or 6 rings, on the other side, Awnless Brome could accumulate more 2-4 rings PAHs than Alfalfa. The activities of PPO in Alfalfa rhizospheric area of sludge were higher than those in Awnless Brome, while it was opposite for DH, which was in match with difference of the removal rates and accumulation contribution rates between the two plants. There were specific microorganisms in high PAHs sludge planted with Awnless Brome and Alfalfa, with which PAHs could be degraded, and more useful bands were found in rhizospheric area sludge of Awnless Brome, which could be the potential reason for the effective degradation with Awnless Brome. Our findings supported the potential use of Awnless Brome for sludge PAHs remediation through its effect on changing activities of enzyme and microbes of sludge in rhizospheric area. Furthermore, based on the removal characteristics, the better way may be intercropping Awnless Brome and Alfalfa, which warrant further studies in the future.

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