Effects of dissolved organic matter derived from forest leaf litter on biodegradation of phenanthrene in aqueous phase

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Abstract

Dissolved organic matter (DOM) released from forest leaf litter is potentially effective for the degradation of polycyclic aromatic hydrocarbons (PAHs), yet the inherent mechanism remains

insufficiently elucidated. In this study, we investigated the effects of DOM derived from *Pinus elliottii* and *Schima superba* leaf litter on the degradation of phenanthrene by the phenanthrene degrading bacterium *Sphingobium* sp. Phe-1. DOM from different origins and at a large range of concentrations enhanced the degradation rate of phenanthrene. DOM derived from *P. elliottii* leaf litter decomposed for 12 months used at a concentration of 100 mg/L yielded the highest degradation rate (16.9 % in 36 h) and shortened the degradation time from 48 h to 24 h. Changes in the composition of DOM during degradation as measured by EEMs-FRI showed that proteins and tyrosine in the DOM supplied readily available nutrients that stimulated biological activity of Phe-1, increasing its growth rate and catechol 2,3-dioxygenase activity. Simultaneously, fulvic acid and humic acid in the DOM enhanced phenanthrene bioavailability by increasing the solubility and mass transfer of phenanthrene, enhancing the uptake kinetics of Phe-1, and increasing the bacteria's direct access to DOM-associated phenanthrene. Humic acid was co-metabolized by Phe-1, resulting in further stimulation of phenanthrene degradation.

Keywords: phenanthrene; microbial degradation; dissolved organic matter (DOM); leaf litter; co-metabolism of HA

1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic pollutants with stable ring structures, poor water solubility, and high octanol-water partition coefficients. PAHs can persist in soils and sediments, where they pose a serious threat to the environment and human health [1, 2]. The forest canopy can

remove PAHs from the atmosphere by interception, adsorption, and absorption, and subsequently the PAHs can return to the soil via wet or dry deposition (including litter fall) [3, 4]. Hence, the soil is the main reservoir for POPs (Persistent Organic Pollutants) including PAHs. It was reported that the yearly fluxes of the PAHs are about 53.3-80 mg/year/m², of which litterfall and throughfall contribute to only 40 µg/year/m² and 213 µg/year/m², respectively [5]. Among the various PAHs, phenanthrene is the dominant one [6], and its concentration in the forest soil is the highest as well [7, 8]. Although microbial degradation is the primary way that PAHs are removed from forest soils [2, 9, 10], this biodegradation occurs to a limited extent in natural environments because of the low bioavailability of PAHs (they have low water solubility and are inclined to adsorb to the soil solid phase) and the low adaptability or activity of microbes [11-13]. Many studies have reported that degradation of PAHs could be enhanced by stimulation of microbial growth through addition of a supplemental carbon source or other microbial nutrients as well as by addition of humic acid (HA), fulvic acid (FA), or a surfactant/bio-surfactant to promote the bioavailability of PAHs by enhancing their apparent solubility and diffusive mass transfer [11, 12, 14, 15]. However, much research in this area focus on adding a single material (such as fulvic acid, humic acid, glucose or peptone) to promote microbial growth or enhance the bioavailability of PAHs, which is, more or less, insufficient to elucidate the potentially complex function of exogenous amendments in the natural environment.

Nevertheless, though the effect of the dissolved organic matters (DOM) on PAHs degradation has been studied in some other researches, the interacting mechanisms remain unclear [16-20]. Up to date, the effect of DOM on the degradation of PAHs was mainly explicated by the multiple actions of DOM

against both PAHs and their degrading microorganisms [21-23]. On the one hand, DOM can form complexes or micelle with PAHs because of its special structure or performance similar to surfactant, thereby affecting their bioavailability and toxicity [24]. Some authors suggested that addition of DOM decreases the solubility and consequently the bioavailability of PAHs, which seems like a drawback in their degradation [25, 26]. However, other researchers concluded that the solubility decrease will not influence the degradation of PAHs because the DOM could bind PAHs and hence increase the diffusive flux of PAHs, making them much more available to bacteria [21-23, 27]. By this way, exemplary cases reported by Ortega-Calvo et al. and Haderlein et al. showed that in the presence of HA-clay in liquid culture, phenanthrene was more rapidly mineralized [21, 28]. On the other hand, to acquire sufficient nutrients, the bacteria will preferentially adsorb to DOM rather than PAHs, which could favor the growth of microbial strains active in biodegradation, and subsequently induce the expression of intermediate degradation enzymes by these strains [29-31]. But it should be noted, though reported in limited literature, that the DOM will also inhibit the cell adhesion to the pollutant source and inhibit the degradation of PAHs [22]. Nevertheless some other studies proved that contaminants adsorbed to mineral-HA are bioavailable, and sorption of both the bacteria and the PAHs to HA stimulates PAHs degradation [23, 28]. From the foregoing, it could be concluded that the influence of DOM on the degradation of PAHs is strongly compound dependent, which is closely related to the binding sites and functioning groups embedded in different compounds of the DOM.

DOM in soil is a mixture or continuum of organic molecules with different structures and molecular weights. In view of its diverse composition, DOM was regarded as part of bio-stimulation

strategies to accelerate the biodegradation of PAHs [9, 32, 33] through its various compounds by influencing either the PAHs bioavailability or the bacterial process. Toyama (2011) [34] proved that the phenolic compounds of *Phragmites australis* root exudates acted as a carbon source supporting growth and induced benzo[a]pyrene-degrading activity of the a *Mycobacterium* strain. Yi (2007) [35] suggested that linoleic acid was the major substance that stimulated PAHs degradation based on comparison of the chemical compositions of the effective plants. Meng (2011) [36] proved that lipophilic extract was the major rhizodeposit enhancing pyrene biodegradation, while water-soluble extract stimulated microbial growth most efficiently. The DOM released from forest leaf litter is an important source of organic carbon in the forest system. However, the mechanisms responsible for these effects are not very clear. For example, which fraction of DOM contributes to the bioavailability of PAHs and/or promote the functioning of the degrading strain, which DOM fractions act on PAHs, and which process come first? How to enhance both the bioavailability of PAHs and the biological activity of degrading bacteria through the use of DOM are questions addressed by the present study, because the best biological stimulation strategy will exploit the synergy between these factors[37].

In the current study, phenanthrene was chosen as a representative PAH (the concentration of phenanthrene in the forest soil is highest among the 16 PAHs in south China [7], our previous study also showed similar result (Fig.S12)), and a bacterial strain (*Sphingobium* sp. Phe-1) with high degradation efficiency was selected by screening bacteria collected from a site highly contaminated by PAHs. DOM was derived from *Pinus elliottii* and *Schima superba* leaf litter that had decomposed in the wild for 0, 2, 5, 8, 12 months. Experiments were conducted to study the effect of DOM on the

degradation of phenanthrene by the Phe-1 strain and to better understand the interacting mechanisms in the DOM-PAH-microorganism system. Excitation-emission matrix fluorescence spectroscopy coupled with fluorescence regional integration (EEMs-FRI) was used to determine the composition of DOM before and after degradation of phenanthrene based on analysis of five excitation-emission regions[38].

2 Materials and methods

2.1 Chemicals

Phenanthrene (TGI, Japan, purity > 97%) was obtained from Aldrich Chemical Co. Phenanthrene-d10 and M-terphenyl (O2Si, USA, radiochemical purity > 99.99%) were supplied by Sigma Chemical Co.

2.2 Culture medium

The *Sphingobium* sp. Phe-1 strain used in the present research was capable of utilizing phenanthrene as a source of carbon and energy, as determined using minimal medium (MM) containing, in 1 L Milli-Q water (Millipore Massachusetts, USA): 3 g (NH₄)₂SO₄, 0.5 g KH₂PO₄, 0.5 g Na₂HPO₄, 0.2609 g MgSO₄, and 1 mL trace elements, pH 7.0. The trace element solution contained, per liter: 0.3 g FeCl₃, 0.3 g FeSO₄·7H₂O, 0.15 g MnSO₄·H₂O, 0.14 g ZnSO₄, and 0.2 g CoCl₂ [39]. The MM was sterilized by autoclaving for 30 minutes at 121 °C using an ALD CL-32L Autoclave (Kyoto, Japan) and was cooled to room temperature before use.

2.3 Bacterium

A phenanthrene-degrading bacterium, *Sphingobium* sp. Phe-1, with high degradation efficiency was selected by screening bacteria collected from a site highly contaminated by PAHs near a refinery in Qingyuan, Guangdong province. According to the literature [40], soil suspension was prepared by placing 10 g fresh soil in 90 mL Mill-Q water and shaking for 30 min at 180 rpm at 30 °C. Then 1 mL soil suspension was inoculated into 250 mL Erlenmeyer flask containing 99 mL of sterilized MM supplemented with 1 mg of phenanthrene and incubated aerobically for 5 d at 30 °C at 160 rpm. Subsequent incubations and isolations were conducted by following the same procedure as the literature [40], but the amount of phenanthrene was increased gradually by 1, 5, 10, 20, 25 mg. Further identification was carried out by phylogenetic analysis followed by the sequencing of 16S rDNA. The Phe-1 strain incubation conditions was investigated [40] and the best growth condition of Phe-1 was achieved with 100 mg/L phenanthrene in a minimal medium at pH 7.0, with shaking at 160 rpm and a temperature of 35 °C.

2.4 DOM

Pinus elliottii and *Schima superba* were chosen as representative needle-leaved and broad-leaved tree species. Approximately 10 g of leaf litter of one species was spread evenly in a 20 cm × 20 cm mesh litter bag with a 2.540-mm mesh upper surface and a 0.635-mm mesh lower surface. The bags were placed at locations adjacent to foliage collection sites in a *P. elliottii* plantation with 20 m × 20 m spacing or a *S. superba* plantation with 15 m × 15 m spacing in March 2013. The leaf litter

was allowed to decompose in the field for different periods of time (0, 2, 5, 8, and 12 months). After degradation, samples were gently rinsed with Milli-Q water, air-dried, and cut into 0.5-cm pieces. DOM was extracted from the decomposed litter by mixing litter and Milli-Q water at a litter to water ratio of 1:10 (m/v). This mixture was shaken for 24 h at 200 rpm on a horizontal rotator, centrifuged for 15 min at 8000 rpm at 4 °C, and then filtered through a 0.2- μ m cellulose acetate membrane (Sartorius CA/0.2 μ m Göttingen, Germany). The leached DOM was characterized and used in degradation experiments within one week [41, 42]. Additionally, the concentrations of the 15 congeners of PAHs in the DOM were detected in a preliminary study (Fig S11) and they were proved too low to affect the phenanthrene degradation efficiency discussed in this current study.

2.5 Experimental approach

Three substudies were carried out to determine the effects of DOM with different properties and concentrations on the degradation of phenanthrene by Phe-1 and to investigate the mechanisms of these effects.

2.5.1 Influence of litter species, decomposition time, and DOM concentration on degradation of phenanthrene

DOM derived from *P. elliotii* (PD) and *S. superba* (SD) leaf litter that had decomposed in the field for 0, 2, 5, 8, or 12 months was used in a phenanthrene degradation experiment. The experiment was performed with 30-mL mixture of minimal medium (MM) containing DOM (SD at 0, 50, 100, 200, and 400 mg/L; PD at 0, 50, 100 mg/L), the strain Phe-1 (10^9 cells/mL) and 100 mg/L phenanthrene in a 50-mL Erlenmeyer flask with a permeable silica gel stopper (phenanthrene dissolved in hexane was

first added to the dry flask, and then MM was added after the hexane had completely vaporized). The suspension was incubated at 35 °C on an orbital shaker (THZ-C, Guangzhou, China) operating at 160 rpm for 36 h, after which the residual phenanthrene was extracted by methylene chloride and measured by GC-MS. Treatment setting is in Table.1.

For each species and litter decomposition time, the DOM concentration (100 mg/L) that had a greatest promoting effect on phenanthrene degradation was used in a second series of degradation experiments under the conditions described above. The growth (OD600) and catechol 2,3-dioxygenase (C23O) activity of Phe-1 were measured.

2.5.2 Mechanisms of DOM influence on phenanthrene degradation

DOM derived from *P. elliotii* (PD) and *S. superba* (SD) leaf litter that had decomposed in the field for 0, 2, 5, 8, or 12 months was used at the concentration of 100 mg/L. Controls that lacked either phenanthrene (DP1) or Phe-1 cells (DP) were set. At the end of the degradation experiment (the incubation conditions were identical to those in 2.5.1), DOM composition was characterized by EEMs-FRI and pH was determined.

Using the DOM that had exhibited the best phenanthrene degradation performance in previous experiments (P12), another series of degradation experiments was carried out by setting P12 at different concentrations of 0, 1, 50, 100, and 200 mg/L. The incubation conditions were identical to those in 2.5.1. Outcomes measured were solubility and percent degradation of phenanthrene, and growth (OD600) and C23O activity of Phe-1.

2.5.3 Degradation time course of phenanthrene in the presence of DOM

A phenanthrene degradation time course experiment with incubation conditions identical to those in 2.5.1 was carried out using P12 at a concentration of 100 mg/L under the conditions described above (2.5.1), with controls that lacked either phenanthrene (DP1) or DOM (PP). Percent degradation of phenanthrene, pH, and Phe-1 growth and C23O activity were determined at incubation times of 0, 12, 24, and 36 h. In addition, composition of DOM composition before and after degradation was analyzed by EEMs-FRI.

2.6 Analytical methods

OD600 and C23O activity of Phe-1 were measured by Shimadzu UV-Vis spectrophotometer (Shimadzu UV-1800 Kyoto, Japan) by following methods described in literatures [43, 44], with minor modification by setting the change rate of absorbance at 375 nm (A_{375}) which represent the C23O activity, $A_{375} = (A_2 - A_1) / A_1$. Dissolved organic carbon (DOC) was measured using a TOC-VCSH analyzer (Shimadzu ON-LINE TOC-VCSH Kyoto, Japan). Composition of DOM was analyzed by EEMs-FRI. Fluorescence EEM measurements were conducted with a fluorescence spectrophotometer (Aqualog-UV-800, Horiba, USA), which used a xenon excitation source with slits set to 5 nm for both excitation and emission. The excitation wavelengths were set from 200 to 400 nm in 5-nm increments, and the emission wavelengths were detected from 290 to 500 nm in 2-nm steps [41]. All samples were acidified to pH 3 with HCl and diluted to a final DOC concentration of 10 mg/L. Residual phenanthrene was measured by a gas chromatograph (Thermo-Ultra Trace GC-DSQ Massachusetts, USA) equipped with a mass spectroscopy detector with a quadrupole analyzer. Briefly, cultivating

culture derived at the end of the degradation were put into a 60 mL separatory funnel and the 50 mL Erlenmeyer flask used for cultivation was washed by 7 mL methylene chloride three times to guarantee the phenanthrene left in the Erlenmeyer flask is fully taken into the separatory funnel (the samples were spiked with 2 mg/L phenanthrene-d10 to determine the phenanthrene recovery rate). Subsequent extraction, purification and detection on GC-MS were conducted according to literature [45].

2.7 Statistical methods

Phenanthrene concentrations were quantified using M-terphenyl as an internal standard and adjusted based on the recovery rate of phenanthrene-d10, after which the phenanthrene degradation rate (percent) was calculated. Analysis of variance (ANOVA) was performed to determine whether DOM source, concentration, incubation time, and DOM composition before or after degradation significantly affected phenanthrene degradation. Correlation analysis was performed to measure correlation between the phenanthrene degradation rate and pH, growth and C23O activity of Phe-1, and DOM composition. Correlation coefficients were calculated using Spearman's correlation. ANOVA and correlation were conducted using SPSS 17.0 [46], and figures were drawn using OriginPro8.0 [47]. Robability levels of $P < 0.05$ and $P < 0.01$ were regarded as statistically significant [46].

3 Results and discussion

3.1 Influence of litter species, and litter composition time and DOM concentration on degradation of phenanthrene

3.1.1 Litter species

Organic carbon plays a key role in the environmental fate of PAHs [48]. DOM released from forest leaf litter is an important source of organic carbon in the forest system and can be utilized by microorganisms that degrade PAHs. In the study, the DOM derived from *P. elliotii* and *S. superba* leaf litter allowed to be decomposed in the field for 0, 2, 5, 8, and 12 months promoted faster phenanthrene degradation compared to the control after 36 h of incubation (Fig. 1a and 1b). The DOM derived from *P. elliotii* leaf litter (PD) increased phenanthrene degradation by 8.0-16.9 %, which were generally more than 10% except that in P8 (8.0 % in 100 mg/L, Fig.1b). Meanwhile, the promoting effect of DOM derived from *S. superba* leaf litter (SD) was 1.1-9.7 % (Fig.1a).

The added DOM also enhanced the biological activity of Phe-1, increasing growth and expression of catechol 2,3-dioxygenase (C23O), an enzyme necessary for phenanthrene degradation (Fig. 1c and 1d). DOM also promoted growth and catechol 2,3-dioxygenase activity of the Phe-1 strain (Fig. 1c and 1d). Again, these effects were greater for PD than for SD, which may explain why the former was more effective at promoting phenanthrene degradation. Phenanthrene is cleaved and converted to 1-hydroxy-2-naphthoic acid by catalyzed dioxygenase, after which it divides into two metabolic pathways, one leading to phthalic acid and the other leading to salicylic acid, back to catechol, and then to gentisate before mineralization to water and carbon dioxide [49-51]. Of the two

catechol degradation enzymes catechol 2,3-dioxygenase (C23O) and catechol 1,2-dioxygenase (C12O), only the former showed activity in Phe-1, indicating that the C23O pathway was used. Therefore, C23O activity during phenanthrene degradation was used as an index of biological activity of the Phe-1 strain. Growth of Phe-1 was higher in the DPP treatment (DOM+phenanthrene+Phe-1) compared to the DP1 and PP treatments, showing that DOM and phenanthrene act synergistically to promote the growth of Phe-1 (Fig. 1c).

3.1.2 Litter decomposition time

The results described above showed that DOM increased the degradation rate of phenanthrene. To investigate the changes in DOM during the decomposition time, the EEMs-FRI method was used. Chen (2003)[38] operationally defined five EEM zones such that peaks in different zones represent different types of material. Peaks in zones 1 and 2 indicate the presence of simple aromatic amino acids such as tyrosine[52], peaks in zone 3 indicate fulvic acid-like material (FA-like), peaks in zone 4 are associated with soluble microbial byproduct-like material, and peaks in zone 5 indicate humic acid-like (HA-like) material (Fig. S3).

In the present study, the composition and material of DOM derived from litter that had decomposed for 12 months in the field changed as litter decomposition time increased (Fig.S4a and S5a) as expected. EEM zones 1, 2, and 4 decreased and zones 3 and 5 increased as the leaf litter decomposed, increasing the proportion of HA-like material. Moreover, our previous studies have shown that the degree of aromatization and the molecular weight of DOM increased as litter decomposition time is extended (Fig.S4b, 4c and S5a, 5c). The specific ultraviolet absorbance of SD

at 254 nm (SUV-A254) and 280 nm (SUV-A280) increased with decomposition time, which is evidence of an increase in the aromaticity of DOM (Fig.S4b).

The decomposition time significantly affected the degradation rate for SD but not for PD (Fig. 1a and 1b), and the treatment combination that was most effective was *P. elliotii*-derived DOM (PD) prepared from litter that had decomposed for 12 months (P12) and used at a concentration of 100 mg/L, which enhanced the degradation rate by 16.9 % (Fig. 1b). For the *S. superba*-derived DOM (SD), the best results were also obtained with a litter decomposition time of 12 months (S12) and a SD concentration of 100 mg/L, which enhanced the degradation rate by 9.7 % (Fig. 1a). This effect may have been due to humic materials, because the HA and FA content of the DOM increased as litter decomposition time was extended, while the content of simple proteins and tyrosine decreased (Fig.S4a and S5a). Hur et al. showed that after 90 days of leaf decomposition, the aromaticity of extracted DOM increased and polar components decreased, enhancing nonlinear binding sites that could bind with hydrophobic organic contaminants (HOC), and that microbial changes in DOM may make hydrophobic structures more accessible to phenanthrene, with the result that microorganisms preferentially attack relatively hydrophilic structures of DOM exposed to the aqueous phase [53, 54]. Han et al. demonstrated that residual PAH concentrations in soil were lower after addition of DOM derived from mushroom cultivation substrate waste (MCSW) or DOM derived from cow manure (CM) than after addition of DOM derived from wheat stalks (WS) due to the higher proportion of the hydrophobic fraction in DOM-MCSW and DOM-CM than in DOM-WS[2].

3.1.3 DOM concentrations

The phenanthrene degradation experiment utilizing P12 at different concentrations confirmed that P12 can promote phenanthrene degradation (Fig. 2c) as well as the growth (Fig. 2e) and C23O activity (Fig. 2f) of Phe-1, as was reported above. It also demonstrated that P12 can enhance the solubility of phenanthrene (Fig. 2d). However, it is noteworthy that the curves showing the relationship of these four dependent variables on P12 concentration all had maxima, below which P12 had a promotion effect and above which it had an inhibitory effect (Figs. 2c, 2d, 2e, and 2f). It is possible that low pH or carbon competition between phenanthrene and DOM inhibited C23O activity of Phe-1 (Fig.S7 in P8 and S10 in 200mg/L). Phenanthrene solubility and C23O activity of Phe-1 were highest at an S12 concentration of 50 mg/L, but the phenanthrene degradation rate and growth of Phe-1 were highest at 100 mg/L.

3.2 Mechanisms of DOM influence on phenanthrene degradation

3.2.1 The changes in DOM composition after degradation of phenanthrene

The results described above showed that DOM could enhance the degradation of phenanthrene by promoting the growth and C23O activity of Phe-1 strain and enhancing the solubility of phenanthrene. To investigate the mechanisms by which fractions of DOM influence the degradation of phenanthrene, DOM derived from leaf litter was used in degradation experiments at a concentration of 100 mg/L (Fig.S7 and S8). The results of P12, which was the DOM with the greatest degradation promoting effect, are shown in Fig.2a and 2b.

After 36 h of incubation, zones 3 and 5 decreased in the DP treatment (DOM+phenanthrene) that

lacked Phe-1; zones 1, 2, and 4 decreased in the DP1 treatment (DOM+Phe-1) that lacked phenanthrene; and zones 4 and 5 decreased in the DPP treatment (Fig. 2a). These changes in the composition of DOM provide evidence that the proteins and tyrosine of DOM were used by the strain Phe-1, and that FA and HA in the DOM bound with phenanthrene (then filtered by 0.2- μ m cellulose acetate membrane (CA), resulting in decreasing). The pH observed at the end of the experiment differed between treatments, confirming the EEMs-FRI results (Fig. 2b). During phenanthrene degradation, the pH in the DPP and DP treatments declined due to the HA and FA that remained after utilization of proteins and tyrosine for microbial growth. In addition, HA was co-metabolized during phenanthrene degradation in the DPP treatment (Fig. 2a) (phenanthrene was not detected on CA, thus, the HA would not be filtered) and since the pH of FA is lower than that of HA, the post-incubation pH was lower in the DPP treatment than in the DP1 treatment (Fig. 2b).

We suggest that the proteins and tyrosine of DOM are readily available carbon and nitrogen sources that support growth and C23O expression of Phe-1, and that FA and HA formed complexes with phenanthrene that increased its availability, both of which enhanced the phenanthrene degradation rate. The use of amendments to enhance the degradation of HOCs has been reported previously. Adding peptone, glucose, and yeast extracts increased the rate of microbial degradation of phenanthrene [55], and low-molecular-weight organic acids (LMWOAs), Tween-80, FA improved the bioavailability of PAHs (pyrene and phenanthrene) and increased the degradation rate [18, 56, 57]. Lu (2011) [10] reported that the addition of gaseous toluene as a co-substrate promoted the growth of degrading bacteria and improved degradation enzyme activity, thereby promoting phenanthrene

degradation. Han (2015) [2] showed that DOM derived from agricultural wastes enhanced the mobility of PAHs and increased nutrient levels for indigenous soil microorganisms.

3.2.2 Degradation time course of phenanthrene in the presence of DOM

The results described above show that the FA and HA of DOM increased the bioavailability of phenanthrene and the proteins and tyrosine of DOM promoted the biological activity of Phe-1. However, which of the abovementioned processes came first was not yet determined. In a phenanthrene degradation time course experiment carried out in the presence of 100 mg/L P12, both the phenanthrene degradation rate and growth of Phe-1 were higher in the DPP treatment compared to the PP control at the 12-h time point (Fig. 3a and 3b), indicating that DOM can simultaneously promote Phe-1 growth and phenanthrene availability. Moreover, the sustained high growth between the 12-h and 24-h time points exhibited by Phe-1 in the DPP treatment compared to the DP1 and PP controls (Fig. 3b) implies that other DOM fractions besides proteins and tyrosine can support Phe-1 growth. In addition, the presence of DOM shortened the degradation time from 48h to 24h (Fig. 3a, Fig.S1). Changes in the composition of DOM during the course of phenanthrene degradation were similar to those described above. At 12 h, zones 1, 2, and 4 decreased in the DP1 treatment (Fig. 3d) and zones 2, 4, and 5 decreased in the DPP treatment (Fig. 3e). For the latter treatment, zone 5 decreased at 24 h and 36 h mainly (Fig. 3e).

In the current study, we also found that HA and FA in the DOM could not be utilized by the degrading bacteria in the absence of phenanthrene, but HA could be utilized when phenanthrene was present (Fig. 2a). Based on the EEMs-FRI results (Fig. 2a), we speculate that early in the course of

degradation (Fig. 3e), HA and FA acted as carriers, complexing with phenanthrene and increasing its availability to Phe-1. By the 12-h time point, the FA will have lost its phenanthrene and the HA will be filtered, and between 12 and 24 h HA will be co-metabolized with phenanthrene. The observed changes in pH (Fig. 2b and 3f) confirmed the results of EEMs-FRI. Post-degradation, the pH in the DPP and DP1 treatments had declined due to the presence of residual HA and FA after microbial utilization of DOM proteins and tyrosine. In addition, HA was co-metabolized in the DPP treatment, and since the pH of FA is lower than that of HA, the pH was lower in the DPP compared to the DP1 treatment (Fig. 2b). As shown in Fig. 3f, the pH of both DPP and DP1 declined between 0 h and 12 h, with DPP having a steeper decline. The observed changes in the phenanthrene degradation rate and in Phe-1 biomass over time are additional evidence of co-metabolism of HA. There was little change in the population size of Phe-1 (Fig. 3b) or in the composition of DOM (Fig. 3d) after 12 h in the DP1 treatment because the proteins and tyrosine of DOM were used up, but in the DPP treatment the phenanthrene degradation rate (Fig. 3a) and the biomass of Phe-1 (Fig. 3b) kept increasing after 12 h, and a faster rate than in the PP treatment, indicating that there were substrates other than phenanthrene or the proteins and tyrosine of DOM that were stimulating growth of Phe-1. This is a surprising finding of this study that the HA of DOM could be co-metabolized during phenanthrene degradation.

3.2.3 Interacting mechanisms in the DOM-phenanthrene-microorganism system

Much research has been conducted on the degradation of phenanthrene. Some authors have suggested that microorganisms are able to degrade only dissolved PAHs, and that addition of DOM

decreases the bioavailability of PAHs [25, 26]. Other researchers have concluded that PAHs bound to DOM are accessible to bacteria and can be degraded as rapidly as PAHs in the liquid phase [21, 23, 27].

DOM can form complexes with PAHs by $\text{NH}-\pi$ and $\pi-\pi$ interactions and via hydrophobic and micellar effects, thereby affecting the bioavailability (solubility and diffusive mass transfer) and toxicity of PAHs [9, 24, 33]. DOM, supplied as nutrients, could enhance the biological activity and the uptake rate of bacterium as well [22, 29]. In the present research, the addition of DOM derived from leaf litter significantly hastened the degradation of phenanthrene (Fig. 1a and 1b), and the time of degradation was shortened from 48 h to 24 h (Fig. 3a and Fig. S1). The growth (Fig. 1c, 2e and 3b) and the biological activity (biomass and C23O activity) of Phe-1 (Fig. 1d, 2f and 3c) were increased in the presence of DOM because the proteins and tyrosine of DOM (Fig. 2a) served as sources of readily available carbon and nitrogen for Phe-1 growth before 12 h (Fig. 3b, 3d). The HA and FA of DOM (with more hydrophobic structures more accessible to phenanthrene) affected the fate of phenanthrene primarily by combining with it and increasing its bioavailability at the beginning (Fig. 2a, 2d). Both of that effect acted at the same time. At 12h, the FA have lost its phenanthrene and the HA will be co-metabolized and further stimulate phenanthrene degradation (Fig. 3a, 3b, 3e).

The observed enhancement of phenanthrene degradation could not be explained solely by improved phenanthrene solubility or by the more rapid uptake of phenanthrene by Phe-1 cells. A DOM concentration of 50 mg/L resulted in the highest levels of phenanthrene solubility (Fig. 2d) and C23O activity (Fig. 2f), but the highest degradation rate was observed in the 100 mg/L treatment (Fig.

2c). The solubility of phenanthrene was lower in the 100 mg/L DOM treatment than in the control treatment without DOM (means more absorbed phenanthrene in 100mg/L treatment) (Fig. 2d), which could be explained by the fact that the addition of DOM can reduce substrate concentrations at the surface of microbial cells by enhancing the uptake rate of dissolved compounds and the mass transfer of PAHs on the one hand [58]. On the other hand, the strain phe-1 could use the bound phenanthrene directly. For example, HA (a form of DOM) which has been described with a micellar microstructure similar to that of surfactants, can incorporate dissolved PAHs [12], and a fraction of micellar-phase PAHs is directly bioavailable to cells and does not need to be dissolved in the liquid phase [59]. DOM with many hydrophobic and nonpolar components may be more effective for biodegradation of PAHs because degrading bacteria can access to the incorporated DOM-PAHs directly, and the bioavailability of DOM-PAHs is higher than that of dissolved PAHs [2, 22, 32]. What is more, HA can transport phenanthrene towards the degrading bacteria as well, which enhances their ability to take up both dissolved and bound PAHs [60, 61].

These results suggest that DOM enhanced phenanthrene bioavailability by increasing the solubility and mass transfer of phenanthrene, enhancing the biological activity and the uptake kinetics of Phe-1, and increasing the bacterium's direct access to DOM-bound phenanthrene.

4 Conclusions

In conclusion, we investigated the effects of DOM derived from the leaf litter of two tree species (*Pinus elliottii* as needle-leaved species and *Schima superba* as broad-leaved species) on

microbial degradation of phenanthrene. We found that DOM promoted the degradation of phenanthrene through simultaneous promoting effects on phenanthrene bioavailability and on the biological activity of the bacterium *Sphingobium* sp. Phe-1. Readily available carbon and nitrogen sources in DOM (such as protein and tyrosine) supported growth of Phe-1 and increased the activity of catechol 2,3-dioxygenase, an enzyme in the phenanthrene degradation pathway. FA (fulvic acid) and HA (humic acid) in the DOM absorbed phenanthrene, enhancing its mobility while allowing the bacteria directly access to DOM-bound phenanthrene. HA was co-metabolized by Phe-1, which further stimulated phenanthrene degradation.

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6 Supplementary material

Additional figures and table show measurement data from this study.

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Table 1 Experimental treatment

Treatment	Code	Remarks
DOM derived from <i>S. superba</i>	SD	S0, S2, S5, S8, S12
DOM derived from <i>P. elliotii</i>	PD	P0, P2, P5, P8, P12
DOM + phenanthrene + Phe-1	DPP	treatment with DOM
DOM + Phe-1	DP1	control without phenanthrene
DOM+ phenanthrene	DP	control without bacteria
phenanthrene + Phe-1	PP	control without DOM
phenanthrene + Phe-1 + NaN ₃	PPN	control
PD + phenanthrene + Phe-1	PDPP	treatment with PD
SD + phenanthrene + Phe-1	SDPP	treatment with SD
PD + Phe-1	PDP1	control without phenanthrene
SD + Phe-1	SDP1	control without phenanthrene
PD + phenanthrene	PDP	control without bacteria
SD + phenanthrene	SDP	control without bacteria

Fig.1 Influence of litter species, decomposition time, and DOM concentration on degradation of phenanthrene

Fig. 1 The influence of DOM derived from *P. elliotii* (PD: P0, P2, P5, P8, P12) and *S. superba* (SD: S0, S2, S5, S8, S12) on degradation of phenanthrene (PA). (a) Promotion of PA degradation by SD; (b) promotion of PA degradation by PD; (c) effect of SD and PD on growth of Phe-1; (d) Phe-1 catechol 2,3-dioxygenase (C23O) activity during PA degradation. PDPP, PD+phenanthrene+Phe-1; PDP1, PD+Phe-1; SDPP, SD+phenanthrene+Phe-1; SDP1, SD+Phe-1; PP, phenanthrene+Phe-1; upper case letters indicate significant differences between treatments; lower case letters indicate significant differences between months.

Fig.2 Mechanisms of DOM influence on phenanthrene degradation

Fig.2 Effect of P12 (DOM derived from *P. elliotii* leaf litter that had decomposed for 12 months) on degradation of phenanthrene. (a) change in P12 (at with 100mg/L) composition before and after degradation of phenanthrene measured by EEMs-FRI; (b) effect of treatment on post-incubation pH in P12 with 100mg/L; (c) Phenanthrene degradation rate in different concentrations of P12; (d) solubility of phenanthrene; (e) growth of strain Phe-1 (OD600); (f) catechol 2,3-dioxygenase (C23O) activity of strain Phe-1 (A'375). DP1, DOM+Phe-1; DP, DOM+phenanthrene; DPP, DOM+phenanthrene+Phe-1; C-C0(%), change in DOM content before (C0) and after (C) degradation; zones 1 and 2, aromatic protein and tyrosine; zone 3, fulvic acid (FA); zone4, soluble microbial by-products; zone5, humic acid (HA). **P<0.01; *P<0.05; uppercase letters indicate significant differences between months for SUV-A₂₅₄; lowercase letters indicate significant differences between months treatments.

Fig.3 Degradation kinetics of phenanthrene in the presence of DOM

Fig. 3 Influence of DOM derived from *P. elliotii* leaf litter that had decomposed for 12 months (P12) on the time course of phenanthrene degradation. (a) Phenanthrene degradation rate; (b) Growth of strain Phe-1 (OD600); (c) Catechol 2,3-dioxygenase (C23O) activity of strain Phe-1 (A'375); (d) Change in DOM composition in treatment DP1; (e) Change in DOM composition in treatment DPP; (f) pH in different treatments. DP1, DOM+Phe-1; DPP, DOM+ phenanthrene+Phe-1; PP, phenanthrene+Phe-1; C-C0(%), difference in DOM content before (C0) and after (C) degradation; zones 1 and 2, aromatic proteins and tyrosine; zone 3, fulvic acid (FA); zone 4, soluble microbial by-products; zone 5, humic acid (HA). **P<0.01; *P<0.05; uppercase letters indicate significant differences between treatments; lowercase letters indicate significant differences between concentrations.