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ORIGINAL PAPER

The negative interaction between the degradation of phenanthrene and tricyclazole in medium, soil and soil/compost mixture

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Abstract To assess the co-catabolism of phenanthrene and tricyclazole in different samples, the interaction during the degradation of phenanthrene and tricyclazole were investigated in medium, soil and soil/spent mushroom compost (SMC) mixture. Generally, tricyclazole showed a negative influence on the activity of phenanthrene dioxygenase and it inhibited the degradation of phenanthrene prominently, both in cultures of phenanthrene catabolic isolates (*Sphingomonas paucimobilis* ZX4 and the mixed flora M1) and soils, while a similar inhibition caused by phenanthrene was also found for the degradation of tricyclazole in soil/SMC. However, the inhibition effect on phenanthrene degradation was eliminated in soil/SMC mixture, due to the abundant microorganisms and enormous catabolic potential in SMC. Furthermore, it was proved that the negative influence between phenanthrene and tricyclazole was most likely derived from the molecular similarity and it tended to decrease with improved microbial diversity in environment.

Keywords Phenanthrene · Tricyclazole · Inhibition · Degradation · Soil · Spent mushroom compost

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Introduction

As one of polycyclic aromatic hydrocarbons (PAHs), phenanthrene is a widespread environmental pollutant and has been widely concerned due to its toxic property as well as high tendency of bioaccumulation in organisms (Halling-Sorensen et al. 2000; Shailaja and Rodrigues 2003). The degradation of phenanthrene in contaminated environments has attracted great attention and a lot of methods have been developed to diminish this pollution in last decade. However, the knowledge about co-catabolism and interaction between phenanthrene and other organic compounds in environments is still poor despite of the diversity of pollutants in environments. Several reports have investigated the co-catabolism between different PAHs and their corresponding heterocyclic PAHs in culture medium (Tiehm and Fritzsche 1995; Lantz et al. 1997) or environments (Meyer and Steinhart 2001), and most of which revealed an antagonistic impact. It remains uncertain, however, whether the negative effects between PAHs and their

analogues in different systems are derived from the competition of molecules or not. Besides the possible interaction, many polycyclic compounds showed considerable toxicity to microorganisms, which slow down the degradation process of the target pollutants. And this negative influence caused by toxic pollutants could not be eliminated in most of studies (Lantz et al. 1997; Meyer and Steinhart 2000). Additionally, further studies are still necessary to investigate difference of the agonistic interaction in various environments.

As a commonly used fungicide, tricyclazole [5-methyl-1, 2,4-triazolo(3, 4-b)benzothiazole] is employed for controlling the rice blast disease in China and can provide rice with long-term protection from this disease during the whole growth process because of its stability and accumulation in soil (Padovani et al. 2006). In other ways, tricyclazole is a fused triheterocyclic compound and has a plane molecule with similar shape as that of phenanthrene (Dong et al. 2002). Nevertheless, there is still no report on its toxicity to microorganisms so far except for an inhibition on formation of fungal pigments (Wheeler and Klich 1995).

In this study, we aim to provide the phenomena and mechanism of the interaction between phenanthrene and tricyclazole through the degradations of pollutants in medium, soil and soil/spent mushroom compost (SMC) mixture, whose high catabolic capability to a wide range of xenobiotic compounds has been proved (Semple et al. 1998; Lau et al. 2003). Furthermore, a potential strategy is proposed to eliminate the possible agonistic influence and accelerate the remediation of contaminated environments where various pollutants with similar structures exist.

Materials and methods

Experimental materials

Phenanthrene was purchased from Fluka (Switzerland, purity > 99%). Tricyclazole (purity > 97%) was from Changhe agricultural chemistry company (HangZhou, China). Test soil was collected from an experimental farm of Zhejiang University, air-dried and sieved through 0.2 mm mesh. Spent mushroom compost (SMC) from Huadan agricultural food Ltd. (Hangzhou, China) was collected after the cultivation of *Pleurotus ostreatus* and mainly consisted of cottonseed and straw. Phenanthrene-degrading strain *Sphingomonas paucimobilis* ZX4 and mixed flora M1 (consists of 5 types of phenanthrene-degrading isolates) were isolated and identified as described by Xia et al. (2003).

Effect of tricyclazole on phenanthrene degrading isolates

Phenanthrene degrading isolates ZX4 and M1 were inoculated in Bushnell–Haas medium (Wreen and Venosa 1996) supplemented with phenanthrene (1,000 mg l^{-1}) and tricyclazole (0, 30, 60, 120 or 250 mg l^{-1}, respectively), three parallel replicates for each tricyclazole concentration. The cultures were incubated at 30°C and 130 rpm in shaker and the growth rate was determined with interval of 12 h photometrically at 600 nm.

Assay of enzymes

Strain ZX4 and mixed flora M1 were inoculated to Bushnell–Haas medium with phenanthrene (1,000 mg l^{-1}) and incubated at 30°C in an orbital shaker. All cells were collected at their exponential growth phase. The activities of phenanthrene dioxygenase (PDO), 1-hydroxy-2-naphthoate (1H2N) degrading enzymes (dioxygenase or hydroxylase), catechol 1, 2-dioxygenase (C12O) and catechol 2, 3-dioxygenase (C23O) were determined with the methods described previously by Chen and Aitken (1999) and Tian et al. (2002). The enzyme activities were estimated with the increase or decrease in absorbance at 300, 375 and 600 nm, respectively, while the protein content was analyzed with Folin-phenol method (Lowry et al. 1951) using bovine serum as standard.

Degradation of phenanthrene and tricyclazole in soil and soil/SMC system

Phenanthrene and tricyclazole were dissolved in acetone and added to soil to a final concentration of 200 and 60 mg kg^{-1}, respectively, then the soils were homogenized and the solvent was evaporated completely in ventilated cabinet. The moisture was adjusted to 17% (w/w), corresponding to 50% of water holding capacity.

All treatments (listed in Table 1) were studied in 1.5 l jars, three replicates each, incubated in dark at 30°C and mixed twice a week, with water amended to maintain appropriated moisture during incubation period. Chemical analyses and microbial enumeration were performed on sampling days 1, 3, 5, 7, 14, 21, 28, 42 and 56, respectively. Approximately 50 g of sample was collected from five different points in each treatment for sampling and used for extraction and microbial enumeration.

Monitoring of PAH-degrading microbial population

Total PAH-degrading microbial population was measured on sampling days with a miniaturized most probable number (MPN) technique, using 96-well microtiter plates as described previously by Wreen and Venosa (1996). The samples were inoculated into Bushnell–Haas medium in wells with a mixture of anthracene, phenanthrene, fluorene and dibenzothiophene acting as carbon source. The color in positive wells would turn yellow or brown during 3-week incubation at room temperature.

Extraction of contaminants

All soils and soil/SMCs were air-dried and grinded to powder, and subsequently 3 g of each sample was extracted according to Eschenbach et al. (1994). The resulting extracts were divided into two fractions, the free fraction from ultrasonic extraction with acetone (phenanthrene) or chloroform (tricyclazole), and the fraction of bound pollutants, generated via saponified extraction with alkali at 68°C for 2 h. The extraction method was checked in paddy soil, garden soil, SMC, and the average extraction efficiency in this study reached up to 91 ± 4.9% (for phenanthrene) and 93.7 ± 7.5% (for tricyclazole).

HPLC analysis

The purified extracts were analyzed by high performance liquid chromatography (Waters, type 2505, USA).

Phenanthrene in each extract section was separated and analyzed under a linear elution gradient of acetonitrile/water from 50:50 (v/v) to 80:20 (v/v) within 18 min at a flow rate of 1 ml min^{-1}. An isocratic elution was carried out for detection of tricyclazole using a mixture of acetonitrile and water (40:60, v/v) at 1 ml min^{-1}. The concentration of phenanthrene and tricyclazole was determined at 250 and 225 nm, respectively. The quantification of total pollutant concentration was done according to the method described previously (Eschenbach et al. 1994).

Statistical analysis

All data were subjected to analysis of *T*-test or ANOVA with SPSS 10.0 (SPSS Inc.) for determination of statistical significance.

Results

Growth of phenanthrene-degrading isolates

Phenanthrene-degrading strain *Sphingomonas paucimobilis* ZX4 was isolated and identified from creosote contaminated soil (Xia et al. 2003). It could

Table 1 Soil and soil/spent mushroom compost treatments

Code	Composition	Treatment
1 ■[a]	1.8 kg contaminated soil	Tricyclazole (60 mg kg^{-1})
2 ●	1.8 kg contaminated soil	Phenanthrene (200 mg kg^{-1})
3 ▲	1.8 kg contaminated soil	Phenanthrene (200 mg kg^{-1}) + tricyclazole (60 mg kg^{-1})
4 □	1.5 kg contaminated soil/SMC (5:1, w/w)	Tricyclazole (60 mg kg^{-1})
5 ○	1.5 kg contaminated soil/SMC (5:1, w/w)	Phenanthrene (200 mg kg^{-1})
6 △	1.5 kg contaminated soil/SMC (5:1, w/w)	Phenanthrene (200 mg kg^{-1}) + tricyclazole (60 mg kg^{-1})

[a] The symbols following the treatment code were the ones used in corresponding graphs

decompose at least 90% of phenanthrene within 10 days in liquid medium via salicylate pathway. The phenanthrene catabolic mixed flora M1 was also isolated in our group, which could remove phenanthrene at a similar rate as strain ZX4, but through both *o*-phthalate and salicylate pathways.

Figure 1 illustrates the growth curves of ZX4 (a) and mixed flora M1 (b) in liquid culture supplemented with phenanthrene and different concentrations of tricyclazole. As shown, strain ZX4 could grow well at the absence of tricyclazole after a 2-day lag phase, reaching its maximal optical density (OD) at day 8, while the growth of ZX4 was significantly inhibited at the presence of tricyclazole (ANOVA, $P < 0.05$). A longer lag phase and lower OD value was observed at a lowest concentration of tricyclazole (30 mg l^{-1}), and no growth was observed with higher tricyclazole concentration.

Without tricyclazole, the mixed flora M1 also got a fast growth in liquid culture and reached the maximal growth within 5 days (Fig. 1b). While M1 barely grew at the presence of tricyclazole except the culture with 30 mg l^{-1} tricyclazole, in which the optical density kept rising after a 6-day lag time and reached its maximal value 5 days later. Besides, a higher OD value recorded in presence of tricyclazole (30 mg l^{-1}) at day 10 implies a partial degradation of tricyclazole by phenanthrene degraders.

As observed with strain ZX4, increasing concentration of tricyclazole also aggrandized the inhibition on catabolism of phenanthrene in M1. When the concentration of tricyclazole in medium was over 60 mg l^{-1}, M1 did not grow until the 11th day, and no discriminable growth was detected in culture with as much as 250 mg l^{-1} of tricyclazole.

It showed that tricyclazole could inhibit the growth of phenanthrene degrading isolates ZX4 and M1 and the agonistic influence of tricyclazole was positively related with its concentration.

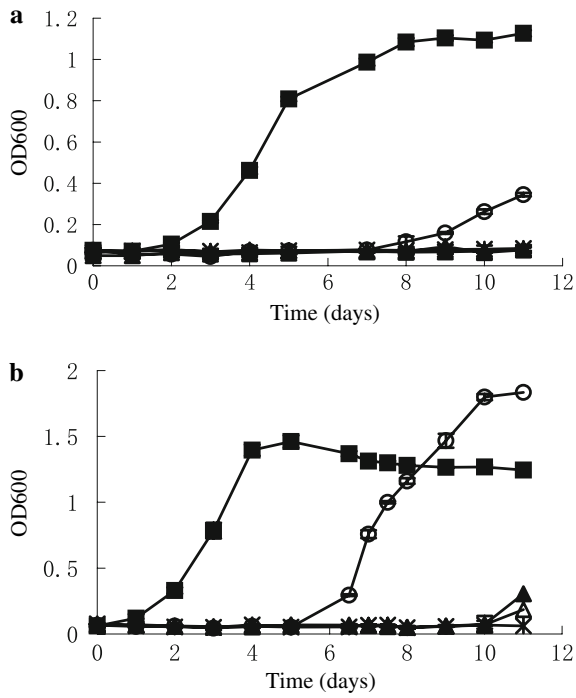

Fig. 1 Growth curves of strain ZX4 (**a**) and mixed flora M1 (**b**) in culture media with phenanthrene (1000 mg l^{-1}) and different concentrations of tricyclazole. ■ (0 mg l^{-1}), ○ (30 mg l^{-1}), ▲ (60 mg l^{-1}), △ (120 mg l^{-1}), * (250 mg l^{-1})

Assay of phenanthrene catabolic enzymes

The PDO, 1H2N degrading enzymes (dioxygenase or hydroxylase), C12O and C23O have been identified as key PAH degradation-related enzymes at upper, middle and lower positions in phenanthrene degradation pathway, respectively (Chen and Aitken 1999; Tian et al. 2002).

Analysis revealed no apparent impact of tricyclazole on 1H2N degrading enzymes, C12O and C23O, but a significant inhibition (ANOVA, $P < 0.05$) on PDO generated by tricyclazole, both in strain ZX4 and mixed flora M1, which might be owing to the structural similarity between substrate (phenanthrene) and inhibitor (tricyclazole). The activity of PDO was reduced by 19.6 ± 2.3% when tricyclazole reached 1/10 (w/w) of total substrate, while 32.5 ± 4.7% and 47.2 ± 4.7% of original PDO was inactivated when the ratio (w/w) of inhibitor to substrate reached 1/5 and 1/3, respectively.

Removal of phenanthrene in soil system

To minimize possible toxicity to microorganisms, 60 mg kg^{-1} dry weight (dw) (w/w) of tricyclazole was applied in soil and soil/SMC mixture, based upon the results of microbial growth and enzyme activities.

The degradation of phenanthrene in contaminated soil systems were presented in Fig. 2. As shown, both

degradation processes could be divided into a lag period and a degrading period, while phenanthrene was more or less removed within 56 days.

During the degrading process of phenanthrene in soil without tricyclazole (treatment 2), the pollutant concentration dropped at an exponential rate ($P < 0.05$, $R^2 = 0.92$) after a 7-day lag phase and 90% of phenanthrene was removed within 4 weeks.

The catabolism of phenanthrene in soil with tricyclazole (treatment 3) was carried out in a significantly different way (T-test, $P < 0.05$), indicating the obvious inhibiting effect from tricyclazole. With this inhibition, phenanthrene was decomposed at a constant rate after the lag phase and its degrading kinetic equation was almost linear ($P < 0.05$, $R^2 = 0.99$), as demonstrated in Fig. 2. Besides, the decomposition of phenanthrene slowed down later on and the removal rate of phenanthrene on 56th day dropped to 72% in treatment 3 while it was more than 95% at the same time in treatment 2. Moreover, an extended lag phase was also observed in degradation of phenanthrene in treatment 3, which was 7 days longer than that in treatment 2.

Additionally, the statistical analyses (T-test) on bound phenanthrene between treatment 2 and treatment 3 revealed no significant impact on the adsorption rate of phenanthrene via tricyclazole. In other words, the presence of tricyclazole could not reduce the concentration of free phenanthrene that was more easily degraded, comparing to the bound one (Nam and Kim 2002).

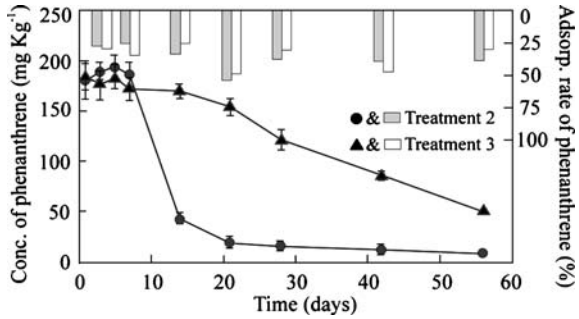

Fig. 2 Degradation of phenanthrene in soil with (treatment 3, 60 mg l^{-1}) or without tricyclazole (treatment 2). The circles and triangles indicate the phenanthrene concentration and columns represent the adsorption rate

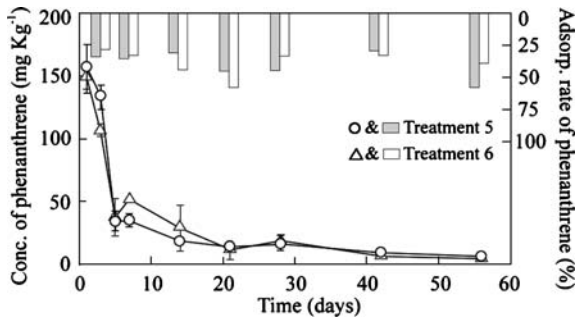

Fig. 3 Degradation of phenanthrene in soil/SMC mixture with (treatment 6, 60 mg l^{-1}) or without tricyclazole (treatment 5). The circles and triangles indicate the phenanthrene concentration and columns represent the adsorption rate

Removal of phenanthrene in soil/SMC system

Figure 3 illustrates the decay of phenanthrene in soil/SMC mixture with (treatment 6) or without tricyclazole (treatment 5). As shown, the concentration of phenanthrene dropped exponentially in both treatments without any appreciable lag phase. Consequently, the half-life of phenanthrene was shortened to 3 days and 95% of phenanthrene was removed within 3 weeks, which was much more effective than that in soil.

Moreover, treatment 5 and 6 had a similar profile of degrading kinetic curves during the decomposition of phenanthrene, and no such noticeable inhibition from tricyclazole was examined in treatment 5 as observed in medium and soil. It seems that SMC could not only improve the removal rate of phenanthrene but also dispel the negative influence of tricyclazole on phenanthrene degradation.

It was also noted that no significant difference among the adsorption rate of phenanthrene in soil and soil/SMC mixture was observed (T-test), despite the abundant humus in SMC. Instead, the addition of SMC only yielded a limited elevation in the amount of bound tricyclazole and the adsorption ratio fluctuated during the remediation process (data not shown), and 40.7 ± 10.9% of tricyclazole could not be eliminated by sorption in soil/SMC mixtures.

The variation in population of PAH-degrading microorganisms in soil and soil/SMC mixture

Figure 4 shows the enumeration of PAH degraders in soil (a) and soil/SMC (b) samples. As shown, the

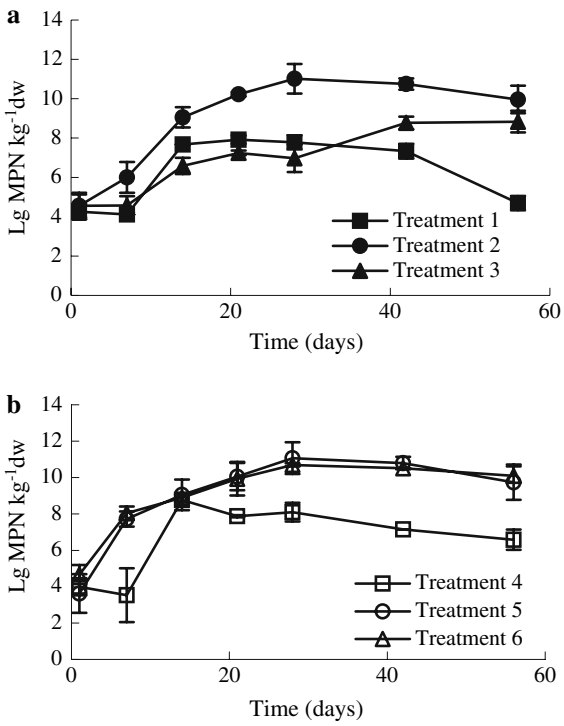

Fig. 4 Populations of PAH-degrading microbes in soil (**a**) and soil/SMC (**b**) samples over the course of 56 days of incubation

population of PAH degraders kept rising in treatment 2 after day 1, subsequently reached the maximal value after 4 weeks and then remained over 10^{10} MPN kg^{-1} dw. However, it varied in a significantly different way in treatment 3 (T-test, $P < 0.05$), with a prominent lag phase and low magnitude level due to the inhibitory effect of tricyclazole.

In contrast to the growth of PAH degraders in media and soil, the degrader population in soil/SMC mixture containing phenanthrene (treatment 5 and treatment 6) rose expeditiously without any appreciable delay and slightly dropped after the maximal growth, suggesting the aptitude of SMC to eliminate the negative effect of tricyclazole.

Effects of phenanthrene on bioavailability of tricyclazole

Generally, tricyclazole is not available for most of degraders in soil, and could persist in soil for at least 8 months (Padovani et al. 2006). It also showed a poor bioavailability in soil in this study; only 10% of

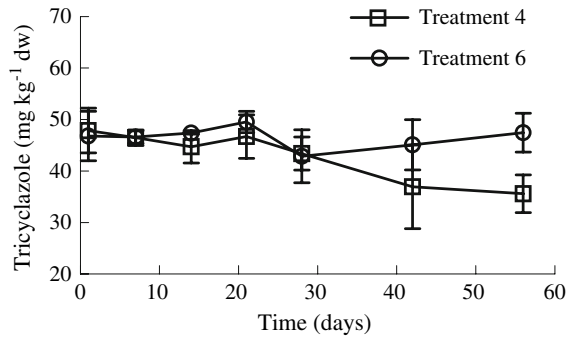

Fig. 5 Degradation of tricyclazole in soil/SMC mixture with (treatment 6) or without (treatment 4) phenanthrene

tricyclazole added was removed in all soils within 56 days. On the other hand, a more progressive degradation was found in tricyclazole contaminated soil/SMC mixture (treatment 4), where 32.8% of tricyclazole was removed during the same period, as illustrated in Fig. 5. Statistical analysis revealed that the addition of SMC could accelerate the degradation of tricyclazole with a linear regression ($P < 0.05$, $R^2 = 0.93$).

Despite all that, the concentration of tricyclazole did not decrease as expected in treatment 6, instead, it only fluctuated around the initial concentration during the degrading process, and no obvious decomposition of tricyclazole was observed. A significant difference in tricyclazole concentrations was found between treatment 5 and treatment 6 after 28 days (T-test, $P < 0.05$), indicating the different catabolism of tricyclazole with or without phenanthrene.

Discussion

In this study, it was observed that both phenanthrene catabolic isolates (ZX4 and M1) could not get normal growth at the presence of tricyclazole in medium, with a notedly prolonged lag phase and obviously reduced growth rate. And none of these two isolates presented a strong capability to degrade tricyclazole. The results of enzymes assay have shown that tricyclazole inhibited activity of PDO (phenanthrene dioxygenase), the key enzyme to initialize the degradation process in phenanthrene catabolic bacteria (Chen and Aitken 1999), whereas the activities of 1H2N degrading enzymes, C12O and C23O activities

were not affected by tricyclazole, suggesting the inhibition was most likely derived from the partial molecular similarity of inhibitor to enzyme substrate. Moreover, a possible explanation was suggested by Andersson et al. (1996), who proved tricyclazole exhibited an inhibition on 1,3,8-trihydroxynaphthalene reductase via competitive binding the active sites in enzyme. Through its agonistic impact on phenanthrene catabolic enzymes, tricyclazole also inhibited the growth of PAH degraders in soils, which acted as the dominant factor for removing phenanthrene (Roling-Wilfred et al. 2002). In treatment 3 (soil with phenanthrene and tricyclazole), as expected, the decomposition of phenanthrene slowed down and the degradation lag phase was prolonged.

All these results show an agreement to previous reports, supporting a wide negative influence on phenanthrene mainly contributed by competition between analogous molecules, either in medium (Tiehm and Fritzsche 1995; Lantz et al. 1997) or environments (Millette et al. 1995). However, the addition of tricyclazole in soil/SMC mixture did not cause a similar inhibition, instead, an enhanced degradation of phenanthrene was observed in all soil/SMC samples here, demonstrated a different result with previous study (Meyer and Steinhart 2000).

It was noticeable that 19 types of hetero-PAHs and PAHs were adopted in Meyer's report and the content of total pollutants nearly doubled when the PAH analogues were added. These pollutants, especially some hetero-PAHs, had remarkable toxicity to microbes, so the presence of inhibition might be partly due to the increase of the toxicity from contaminants. On the contrary, tricyclazole presented no perceptible toxicity to typical bacteria (*Escherichia coli* and *Bacillus subtilis*) and phenanthrene catabolic isolates (ZX4, EVA17 and M1) (Xia et al. 2003, 2005) in our previous work (data not shown).

However, it should be note that no toxic degradation inhibitor of phenanthrene was set as control in this study to estimate the influence of toxicity on the degradation of phenanthrene. Not withstanding their limitation, our results can clearly indicate an agonistic interaction between phenanthrene and tricyclazole, which was most likely derived from the molecular similarity.

Furthermore, a comparison between the microbial diversity and removal rate of phenanthrene in several samples was listed in Table 2, in which the microbial diversity index (Shannon–Weaver index, H) was calculated on the basis of the number and relative intensity of bands revealed by denaturing gradient gel electrophoresis (DGGE), and a high H value indicates a rich diversity of microorganisms in samples (Ampe and Miambi 2000).

As shown in Table 2, the order of removal rate of phenanthrene with the presence of tricyclazole was ZX4 < M1 < treatment 3 < treatment 6, while the microbial diversity followed a reverse order, with highest abundance in treatment 6. It seemed that the inhibition effect of tricyclazole would decrease with a higher diversity of microorganisms.

The system with high microbial diversity, for example, the mushroom compost, which was proved to contain abundant xenobiotic-catabolic microorganisms and enzymes, is able to decompose a wide range of organic pollutants (Semple et al. 1998; Lau et al. 2003; Šašek et al. 2003) and might promote the phenanthrene degradation with various degrading pathways (Lau et al. 2003). Hence, the decay of phenanthrene and the population of PAH degraders in soil/SMC mixture might suggested that the microorganisms in such a system utilize phenanthrene

Table 2 Removal rates of phenanthrene and microbial diversity in different samples

Sample	Removal rate of phenanthrene within 2 weeks (%)	Shannon–Weaver index (H)[a]
Culture of strain ZX4[b]	Not detected	0
Culture of mixed flora M1	4.3	1.86
Treatment 2	80.0	3.89
Treatment 3	7.7	3.76
Treatment 5	83.1	4.53
Treatment 6	80.2	4.57

[a] Calculated following the strategy proposed by Ampe and Miambi (2000)

[b] Both cultures of ZX4 and M1 supplemented 120 mg l^{-1} of tricyclazole and 1,000 mg l^{-1} of phenanthrene

through other pathways that were not affected by tricyclazole, in which some metabolites might be available for the growth of PAH degraders. And the inhibition of tricyclazole could be reduced or eliminated with improved microbial diversity. For example, when the PDO in phenanthrene catabolic bacteria was inactivated, *Pleurotus ostreatus* in SMC, a white-rotten fungus, could oxidize phenanthrene to *trans*-9, 10-dihydrodiol that would be further decomposed through normal phenanthrene degradation pathway (Bezalel et al. 1997). However, further evidence about the difference in microbial communities in all treatments is needed to support this deduction.

Despite that, it is necessary to note that the abundant microbial diversity also facilitate the elimination of phenanthrene and tricyclazole. With higher *H* value, the decomposition of each pollutant was enhanced in soil/SMC mixture comparing to that in soil, except for the tricyclazole with phenanthrene, whose degradation was heavily inhibited by phenanthrene because of its high basicity (pKa = 1.8) (Meyer and Steinhart 2000). Hence, these results propose a potential application of SMC for remediation of contaminated soil containing various pollutants with similar structures, which shows a little difference with traditional bioaugmentation (Boon et al. 2000; Schwartz and Scow 2001) by improving the catabolic microbe population together with the microbial diversity. As a result, it is predicable that the decomposition of pollutants would be prominently enhanced in such a system and the negative interaction among pollutants could be reduced.

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