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ENVIRONMENTAL BIOTECHNOLOGY

Heavy metals species affect fungal-bacterial synergism during the bioremediation of fluoranthene

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Abstract The co-occurrence of polycyclic aromatic hydrocarbons (PAHs) with heavy metals (HMs) is very common in contaminated soils, but the influence of HMs on fungal-bacterial synergism during PAH bioremediation has not been investigated. The bioremediation of fluoranthene-contaminated sand using co-cultures of *Acremonium* sp. P0997 and *Bacillus subtilis* showed increases of 109.4 and 9.8 % in degradation compared to pure bacterial and fungal cultures, respectively, removing 64.1 ± 1.4 % fluoanthene in total. The presence of Cu^{2+} reduced fluoranthene removal to 53.7 ± 1.7 %, while inhibiting bacterial growth, and reducing translocation of bacteria on fungal hyphae by 49.5 %, in terms of the bacterial translocation ratio. Cu^{2+} reduced bacterial diffusion by 46.8 and 31.9 %, as reflected by D (a bulk random motility diffusional coefficient) and D_{eff} (the effective one-dimensional diffusion coefficient) compared to the control without HM supplementation, respectively. However, Mn^{2+} resulted in a 78.2 ± 1.9 % fluoranthene degradation, representing an increase of 21.9 %, while enhancing bacterial growth and bacterial translocation on fungal hyphae, showing a 12.0 % increase in translocation ratio, with no observable impact on D and D_{eff}. Hence, the presence of HMs has been shown to affect fungal-bacterial synergism in PAH degradation, and this effect differs with HM species.

Keywords Fungal-bacterial synergism · Heavy metals species · Bioremediation · Bacterial movement · Fungal highway

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Introduction

Bioremediation is an efficient tool to transform pollutants such as polycyclic aromatic hydrocarbons (PAHs) to less hazardous/non-hazardous forms in an eco-friendly manner, with less input in terms of chemicals, energy, and time (Haritash and Kaushik 2009). Many attempts to improve degradation of PAHs or other hydrophobic organic pollutants (HOCs) often focus on bioremediation with fungal–bacterial consortia exhibiting synergistic effects in heterogeneous soil environments (Furuno et al. 2010; Husaini et al. 2008). The synergistic degradation from fungal-bacterial interactions has been partly ascribed to the increased dispersal of degradative bacteria along the hyphae of the fungal partner (Boersma et al. 2010; Furuno et al. 2010; Kohlmeier et al. 2005), as mycelial networks can act as "highways," allowing bacteria to overcome motility restrictions in soil and reach remote areas, possibly due to the presence of water films on fungal hyphae, which permits bacterial transport along fungal hyphae (Knudsen et al. 2013; Kohlmeier et al. 2005). Thus, the concept of a "fungal highway" can provide new insight into mechanisms for synergistic biodegradation from fungal-bacterial interaction and could have significant benefits for developing novel bioremediation strategies for PAHs pollution in soils (Banitz et al. 2013).

Fungal-mediated translocation of bacteria has been demonstrated to enhance bioavailability of HOCs for bacteria in soil, and this has been confirmed mostly by common fungi with no PAH degradation ability (Banitz et al. 2013). For example, motile *Pseudomonas putida* PpG7 (NAH7) and fast-growing, hydrophilic *Pythium ultimum* were used as model phenanthrene-degrading and vector organisms, respectively (Wick et al. 2007), in which *P. ultimum* was assumed to possess no PAH (phenanthrene) degradation ability. Additionally, fungal hyphae of *Mortierella* sp. LEJ702, which is another common soil fungus, have also been confirmed to stimulate bacterial dispersion (Knudsen et al. 2013), and common ascomycetes *Fusarium oxysporum* and *Rhexocercosporidium* sp. were confirmed to exhibit a fungal highway mechanism for mobilization of pollutant-degrading bacteria (Kohlmeier et al. 2005). However, these previous investigations concerning the fungal highway for mobilization of pollutant-degrading bacteria were based on the assumption of no fungal degradation, and such model organisms are not typical of actual polluted environments. That is, the presence of common fungi without degradative ability for pollutants do not represent the actual behavior of environmental microorganisms in natural PAH-contaminated soils, as the presence of PAHs in soil may impose a selection pressure and encourage degradative adaption of microbial species. For example, a fungus identified as *F. oxysporum* demonstrating high PAH degradation efficiency has been isolated from a PAH-contaminated farm site, and this isolate has been shown to work synergistically to degrade and mineralize different PAH concentrations with five bacteria in a defined microbial consortium (Jacques et al. 2008). Thus, careful examination of both fungi with PAH degradative ability acting as fungal highways for bacterial dispersal and constructed consortia thereof should shed light on synergistic fungal–bacterial degradation mechanisms in actual polluted environments.

In addition, the co-occurrence of PAHs with HMs is very common in contaminated soils (Thavamani et al. 2012; Thavamani et al. 2011) with 40 % of hazardous waste sites on the Environmental Protection Agency's (EPA) National Priority List (NPL) being co-contaminated with organic pollutants and HMs, for example (Olaniran et al. 2013). While bioremediation of PAHs in soil has been reported to be affected by many environmental factors such as pH and the presence of HMs (Naseri et al. 2014), the presence of HMs on the fungal-bacterial synergism in bioremediation has not been investigated. The presence of HMs coexisting with PAHs may affect bacterial mobilization via fungal transport vectors and subsequently restrict or enhance access of bacteria to contaminants such as PAHs or other HOCs, influencing the final degradation efficiency of these pollutants. However, a lack of understanding of the influence of environmental factors such as HMs on bacterial migration via fungal hyphae remains a critical problem for the development of novel bioremediation approaches based on ecological principles.

The presence of HMs in PAH-contaminated soils showed that a few distinctive species such as fungi or bacteria can emerge through selection pressure to resist several metals (Jianlong et al. 2001; Munoz et al. 2012). Microbes in many co-contaminated sites have developed metal tolerances by forming one or more resistance mechanisms (Olaniran et al. 2013), and exploiting this behavior may improve the removal of organic pollutants from a variety of co-contaminated environments including water, sediments, and soil (Ke et al. 2010; Mielke et al. 2004; Olaniran et al. 2013; Yang et al. 2013). However, these researches have not been extended to the influence of HMs on bacterial dispersion mediated by fungi exhibiting HM resistance and PAH degradation ability, nor their synergistic effect in bioremediation. Clearly, investigations into these influences of HMs within constructed consortia composed of fungus and bacterium with degradation ability are crucial for obtaining the desired enhancement of their PAHs degradation potential.

Using a simple laboratory model system to mimic water-saturated and water-unsaturated porous environments and two microbes including a fungus and a bacterial strain both with HMs resistance and PAH degradation ability, we hypothesized that the presence of HMs may affect the fungal-bacterial synergism in bioremediation through mobilization via fungal transport vectors, depending on metal species. Fluoranthene-degrading strains of the fungus *Acremonium* sp. P0997 and the bacterium *Bacillus subtilis*, which both have resistance to both Cu^{2+} and Mn^{2+} were selected based on the absence of mutual antagonistic effects, as confirmed previously in our laboratory (Ma et al. 2014). This work aims to (i) investigate the synergistic effect of a PAH-degrading fungus-bacterium consortium on fluoranthene degradation, (ii) determine the influence of separate HMs on both the synergistic effect of fluoranthene degradation and the fungus-facilitated bacterial movement, and (iii) elucidate the mechanisms through which HMs affect the synergistic biodegradation effect of fluoranthene. Understanding the impact of biochemical and physical factors on bacterial-fungal interactions and the ecosystem function of contaminant biodegradation would be of high relevance for microbial ecology.

Experimental procedures

Organisms, solutions and culture conditions

The fast growing *Acremonium* sp. P0997 (CCTCC M 2013569) was chosen as model organism with hydrophilic mycelial surfaces demonstrating PAH degradation and resistance to Cu^{2+} and Mn^{2+} (Ma et al. 2014). *B. subtilis* (CCTCC AB 2014248) was used as the bacterial motile strain, which

had also been confirmed to resist Cu^{2+} and Mn^{2+} and degrade PAHs in our laboratory (Ma et al. 2015). The fungal and bacterial cells used as inocula in mobilization experiments were prepared in separate PDA liquid medium and nutrient medium (100 mL) at 28 °C and 160 rpm as described previously (Ma et al. 2015).

Stock solutions of individual $MnSO_4$ and $CuSO_4$, and fluoranthene (HPLC grade) and the individual working solutions were prepared as described in our previous report (Ma et al. 2014). Stock solutions of the individual HMs at a concentration of 5000 mg/L were prepared by dissolving $MnSO_4$ and $CuSO_4$ separately in deionized water. Fluoranthene (HPLC grade) was dissolved separately in n-hexane to obtain a fluoranthene stock solution at a concentration of 500 mg/L. Chemicals used in these experiments were of analytical grade and obtained from Sigma-Aldrich unless otherwise specified.

A heterogeneous column system for investigating fungal-bacterial synergism and microbial mobilization during degradation of PAHs

To test fungal-bacterial synergistic degradation and mobilization experiments, a simple laboratory model system as depicted in Fig. 1 was used, which was comprised of a column from a modified syringe (Ø = 30 mm, h = 120 mm), with three different layers of either sand or glass beads (Ø = 1 mm; China). At the bottom of the column, glass beads submerged in a mineral nutrient medium mimicked a water-saturated environment, with the medium carefully suctioned to the 15-mL marking of the syringes. At the top of the column, 25 g of sterilized pretreated sands (initial water content 10 wt%) were used to represent a water-unsaturated porous environment. Between these two layers, an additional layer of sterile glass beads was added to simulate air-filled spaces in soil and separate the water-unsaturated porous sands from the water-saturated environments (i.e., glass beads submerged in mineral medium), and two layers of gauze were overlaid on the top of the glass beads to prevent the possible combination of sands and glass beads. The syringe columns were covered with sterile aluminum foil at the top and sealed with a 0.2-μm closed membrane filter with a cap at the bottom to inhibit air enter into the syringe, respectively, and then placed in a vertical position during experiments. The mineral medium was designed to be no any carbon and nitrogen source to maintain bacterial survival during experiments (Ma et al. 2014). All sand was pre-washed with 0.1 M HCl and distilled water three times before and after each experiment to avoid possible binding of the metal or organic products. The water content of the sand was determined by removing the sand layer from the columns and determining the loss of water after drying at 105 ° for 3 h.

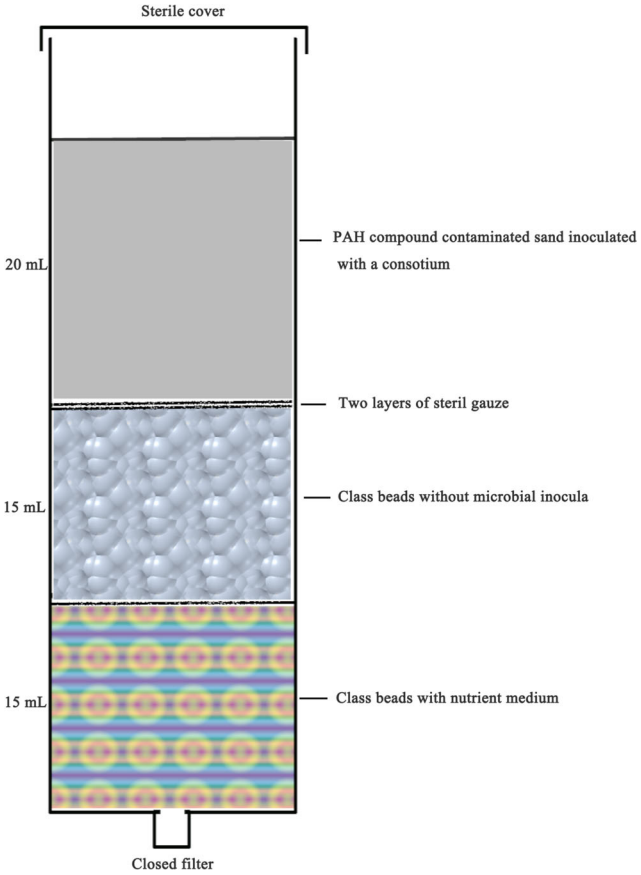

Fig. 1 Schematic view of the experimental setup (modified syringe column, *Ø* = 30 mm, *h* = 120 mm) used for biodegradation and transport experiments mimicking water-saturated glass beads with broth) and air-filled porous soil environments (columns filled with sands). The syringes are placed in a vertical position during experiments

Synergistic PAH degradation and fungal-facilitated bacterial mobilization

For determining the fungal-bacterial synergistic degradation of fluoranthene and the role of fungi in bacterial translocation in heterogeneous soil environments, biodegradation experiments using the design described above were performed in triplicate with the fungus (10 mg wet mycelia/g·sand) or bacteria (0.1 mL broth/g·sand, 10^8 CFU/ mL), or a fungal-bacterial consortium (bacteria 0.05 mL/g; fungus 5 mg mycelia/g·sand) used as inocula. Fluoranthene dissolved in n-hexane was added to the sand, giving a concentration of 80 mg/kg·sand in sand, which had a grain size of 0.8–1.4 mm, water holding capacity (WHC) of 25.2, and was comprised of 99.0 % SiO_2. After evaporation of the n-hexane, the sand was added with individual solutions of urea, KH_2PO_4 and K_2HPO_4 as a basic nutrient supply with a C:N:P ratio of 100:10:1, and mixed with buffered MilliQ water corresponding to 10 % of the water content. After sterilization, the sand was inoculated with different microbial cultures

and placed on the gauze in syringes. Experiments with an identical setup but no inoculum were performed as controls to show the abiotic loss of fluoranthene as well as any contamination of bacteria/fungi.

Synergistic PAH degradation and fungal-facilitated bacterial mobilization in the presence of HMs

To evaluate the effect of HMs on the fungal-bacterial synergistic degradation of fluoranthene and fungal-facilitated bacterial mobilization, experiments were performed using the above experimental setup as shown in Fig. 1 in the presence of Mn^{2+} and Cu^{2+} and inoculated with the same fungal-bacterial consortium. In addition to fluoranthene, the sand was treated with either Cu^{2+} or Mn^{2+} working solution to reach 5 mmol/kg·sand. The experiment was carried out at the same moisture conditions as in the above bioremediation experiments. Experiments in an identical setup inoculated with the same fungal-bacterial consortium but without metal supplementation or without any inoculum but with metal supplementation were performed as controls.

Analysis of bacterial translocation and diffusion

Bacterial or fungal colony forming units (CFUs) in the nutrient broth and in upper sand layer were enumerated by dilution plating and used to describe the dynamics of the co-migration of fungal and bacterial process over distance and time. During experiments, individual vials were sacrificed every 2 days and the cell number in the nutrient broth at the bottom of vials and in the contaminated sand was enumerated. To describe the translocation of bacteria, the fungal-mediated bacterial translocation ratio was calculated as described in Eq. (1).

$$\text{The translocation ratio of bacterial cell} = \frac{\text{Bacterial counts (CFU) in the nutrient broth}}{\text{Bacterial counts (CFU) in the top sand}} \quad (1)$$

We used a previous mechanism of bacterial swimming in liquid films forming along fungal hyphae (Kohlmeier et al. 2005; Wick et al. 2007) to evaluate bacterial diffusion through porous media such as glass beads or sands. As described previously (Knudsen et al. 2013; Kohlmeier et al. 2005; Wick et al. 2007), the observed (one-dimensional) translocation velocity was used to estimate a bulk random motility diffusion coefficient (D). Specifically, the bacterial translocation rate from sand to the broth at the bottom was defined to be the translocation velocity in this study. The one-dimensional form of the Einstein equation (Eq. 2) was deemed appropriate to describe the D of bacterial mobilization on fungal hyphae in the glass-bead pack. Tortuous paths were thought to form by the hyphae as they traverse the glass-bead pack (Ellegaard-Jensen et al. 2014). As determined and confirmed previously (Kohlmeier et al. 2005; Wick et al. 2007), the one-dimensional form of the Einstein equation correlates with the effective one-dimensional diffusion coefficient (D_{eff}) ($\text{cm}^2\ \text{s}^{-1}$), which is related to D by the dimensionless tortuosity τ (Schwarzenbach et al. 1993). D_{eff} relates the mean-squared displacement $< x^2 >$ (cm^2) over a time interval t (s) (Equation 2) (Kohlmeier et al. 2005), in which the observed mean-squared displacement x was defined as the distance from sand at the top to the nutrient media at the bottom (1.8cm, in glass beads; Fig. 1), as well as with t defined as the time needed for bacteria to emerge in the nutrient broth at the bottom, with a typical tortuosity of 1.8 for packed columns containing particles of uniform diameter (glass beads in this experiment) as described previously (Wick et al. 2007).

$$D_{eff} = \frac{D}{\tau} = \frac{< x^2 >}{2\tau} \quad (2)$$

Removal efficiency of fluoranthene

Individual vials were sacrificed after 2, 4, 6, 8, 10, 12, and 14 days, and residual fluoranthene was recovered and analyzed. The removal efficiency of fluoranthene could be calculated as following (Eq. 3), in which C_0 (mg/kg sand) and C_e (mg/kg sand) are the initial and equilibrium concentrations of fluoranthene, respectively. To recover fluoranthene from sand in this study for fluoranthene quantification, an ultrasonic extraction method was used, which was confirmed to recover 99–105 % ($n = 5$, RSD < 5%) of fluoranthene. Fluoranthene analysis was determined by a reverse-phase HPLC on a C_{18} column (ZORBAX Eclipse XDB-C18 4.6 mm × 150 mm, 5 μm, Agilent Technologies) using Agilent 1200HPLC (Japan) equipped with a dual pump and a UV-Vis detector (254 nm) as described in our previous report (Ma et al. 2014).

$$\text{Removal efficiency} = \frac{C_0 - C_e}{C_0} \quad (3)$$

Statistical treatment of the data

All statistical analyses (t tests) were performed using $p < 0.05$ as a criterion for significance. All experiments were performed in triplicate and geometric averages were calculated. Standard deviations were calculated based on real replicates and shown between brackets in the text and/or as error bars in the figures.

Results

The fungal-bacterial synergistic degradation of fluoranthene and mobilization experiments

As shown in Fig. 2, within 14 days of incubation at 28 °a, 30.6 ± 1.4 % of the fluoranthene was degraded by the pure bacterial culture, while the pure fungal culture degraded 58.4 ± 3.1 % fluoranthene. However, a maximum degradation of 64.1 ± 1.4 % fluoranthene was achieved with the fungal-bacterial consortium, yielding increases of 109.4 and 9.8 % compared to pure bacterial and fungal cultures, respectively, and both increases represent a significant difference in degradation efficiency ($p < 0.05$). The degradation efficiency of these inocula increased with time over 14 days after inoculation, and it can be seen that the co-occurrence of the fungus and bacterium resulted in an enhanced biodegradation rate and lower final PAH concentrations compared to pure cultures. This is especially true when comparing consortium degradation to the pure bacterial culture, where the mixed culture degradation was almost twice as high as for bacterial fluoranthene degradation. Although less of an increase in degradation for the mixed culture was observed with respect to the pure fungal culture, it should be mentioned that fluoranthene is one of molecule with a big molecular weight in PAH compounds and difficult to degrade, whereas emphasizing the role of fungi in PAH degradation. The increased degradation by the consortium compared to the pure bacterium may be ascribed the mycelial networks as "highways" for bacteria to overcome motility restrictions and then increase the fluoranthene bioavailability (Banitz et al. 2013; Wick et al. 2007). These results suggest that the consortium consisting of both bacterial and fungal fluoranthene degraders may lead to faster and more complete degradation as compared to single strains and emphasized the role of a fungus with degradation ability in the synergistic fluoranthene degradation, confirming a similar effect observed in previous studies (Furuno et al. 2010; Ma et al. 2015).

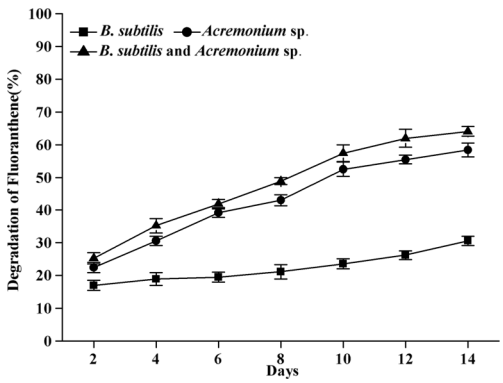

Fig. 2 Degradation of fluoranthene in contaminated sands by *B. subtilis*, *Acremonium* sp., or a mixture of both species in a heterogeneous column system. Data are presented as means ± SE ($n = 3$)

To understand possible mechanisms for this fungal-bacterial synergistic behavior, this study investigated the role of fungal hyphae in bacterial dispersion, using the heterogeneous system described in Fig. 1. Assuming that in moist, water-unsaturated porous environments, the presence of water films on fungal hyphae allow bacterial motility (Wick et al. 2007), bacterial mobilization was estimated by observing the migration of bacteria to un-inoculated mineral media over time. Over the span of 14 days, clearly different results with regard to bacterial mobilization were found in systems inoculated with either pure bacterial or fungal cultures, or a mixed consortium. Only in the shared presence of the fungus and the bacterium were the bacteria observed to be able to cross the air-filled glass layer of 1.8 cm between the fluoranthene-contaminated sand and the medium, which clearly indicated the fungal-mediated translocation of the bacterium from the water-unsaturated sand. In the case of the bacterial inoculation lacking fungi, no indication of mobilization was found, despite the presence of approximately 6.3×10^7 catabolically active bacteria in the contaminated sand, further confirming the role of fungi in bacterial transport. It is likely that the hydrophilic fungal mycelia provide a network of continuous water-pathways for bridging air-filled soil pores, thus facilitating dispersion of motile strain *B. subtilus* as reported previously (Wick et al. 2007), which could in part explain the enhanced biodegradation achieved by the consortium. Thus, these results showed that the presence of fungus with degradation activity facilitated the bacterial dispersal in contaminated sand, confirming that the fungal hyphae act as bacterial transport vectors, which is consistent with previous reports concerning strain mobilization with the help of common fungi with no degradation activity (Banitz et al. 2013; Wick et al. 2007). Together with the results concerning the fungal-bacterial synergistic effect on bioremediation, this study therefore indicates that the application of suitable combinations of catabolically active fungi and bacteria to contaminated soil may be a valuable strategy for enhancing the bioremediation, possibly due to the spreading of bacteria in air-filled soil.

The influence of HMs on the fungal-bacterial synergistic degradation of fluoranthene and bacterial mobilization

The influence of the presence of HMs such as Mn^{2+} and Cu^{2+} on fungal–bacterial synergistic degradation and bacterial mobilization facilitated by fungus was evaluated in the same experimental setups as shown in Fig. 1. PAH degradation, bacteria growth, and the bacteria translocation ratio were determined in the fluoranthene-contaminated sand in the presence of Mn^{2+} and Cu^{2+}. As shown in Fig. 3 A, there is a considerable difference in total fluoranthene degradation with individual supplementations of Mn^{2+} and Cu^{2+} or metal-free ($p < 0.05$). The presence of Mn^{2+} exerted a positive influence on the removal of fluoranthene by the consortium, with a total

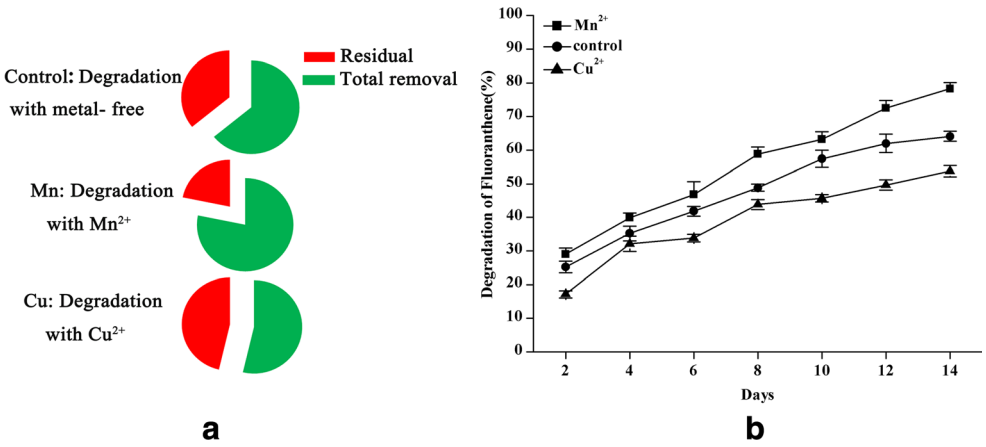

Fig. 3 Degradation of fluoranthene in contaminated sand by a mixture of *B. subtilis* and *Acremonium* sp. in the presence of either Mn^{2+} or Cu^{2+} at 5 mmol/kg·sand in a heterogeneous column system. **a** The total degradation efficiency in different groups. **b** The degradation of fluoranthene with time

removal value of 78.2 ± 1.9 %, representing an increase of 21.9% compared with the metal-free control, which removed 64.1 ± 1.4% of fluoranthene overall. In the case of Cu^{2+}, a significant negative effect on removal was observed, showing an overall decrease in fluoranthene removal efficiency over time, while the presence of the Cu^{2+} reduced fluoranthene removal to 53.7 ± 1.7 %, representing a decrease of 16.2 % with respect to that of control without HM supplementation (Fig. 3b). These results suggest that the presence of HMs affect the fungal-bacterial synergistic biodegradation and the influence differs with the HM species.

Bacterial mobilization and translocation of bacteria on fungal hyphae in the presence of HMs

As seen in Fig. 4a, the presence of individual HMs has a significant effect on bacterial growth in sand with the consortium inoculation, with the presence of Cu^{2+} inhibiting bacterial growth, whereas in the case of Mn^{2+}, bacterial growth was enhanced compared with that in control ($p < 0.05$). As can be seen in Table 1, when the experiments were terminated, there was a noticeable difference in the final colony forming units detected with different HM supplementation compared to the control. Thus, bacterial growth with individual supplementation of Mn^{2+} and Cu^{2+} suggests that the bacterial strain has different resistances to these two HMs, with low resistance to Cu^{2+} and a higher resistance to Mn^{2+} at concentrations of 5 mmol/kg·sand. Also, it was observed that there was no significant difference in the fungal cell weight with the supplementation of individual Mn^{2+} and Cu^{2+} compared with that in the control, showing that this fungus is resistant to these two HMs at a concentration of 5 mmol/kg·sand. As shown in Fig. 4b and Table 1, the bacterial number mobilized in the presence of Mn^{2+} showed an overall increase of 34.4 % and a higher growth rate compared to the control, whereas the presence of Cu^{2+} led to a final mobilization that was 91.8 % lower than the control, when the experiments were terminated. In addition, there was a significant difference in the translocated number of bacteria along fungal hyphae with time over biodegradation, with supplementation of Mn^{2+} showing higher translocation compared to that of Cu^{2+}.

As shown in Fig. 5, there is a significant difference in the bacterial translocation ratio of fungal hyphae with separate supplementations of Mn^{2+} and Cu^{2+} with time of biodegradation ($p < 0.05$); Mn^{2+} supplementation increases the translocation ratio of bacteria whereas Cu^{2+} addition results in a lower increase rate of the translocation ratio of bacteria with time. The value of the translocation ratio of bacteria at all different timepoints during degradation was in the range of 0–60 %, which is comparable with that of the translocation ration of bacteria in a previous report (Furuno et al. 2010). In addition, the ratio of translocation bacteria was not shown to have a linear correlation with the bacterial growth in sand in the presence of HMs during degradation, reflecting the influence of the presence of HMs on bacterial mobilization through the fungal hyphae. The final translocation ratio of Mn^{2+} supplementation was 33.89 %, representing an increase of 12.0 % compared to the control in the absence of HMs, and Cu^{2+} addition resulted in a translocation ratio of 15.5 %, which reflects a decrease of 49.5 % with respect to that of the control. As no significant difference in the fungal cell weight was observed with the supplementation of individual Mn^{2+} and Cu^{2+} and in the control, these results suggest that the presence of HMs in porous media impose a favorable or negative effect on the capacity of fungi to serve as vectors for the dispersion of bacteria, thereby possibly affecting the bioavailability of HOCs for soil bacteria (Fig. 4c).

The influence of the presence of HMs on bacterial diffusion

To further elucidate the role of HMs on the bacterial mobilization, the influence of HMs on bacterial diffusion through porous media such as glass beads was investigated via the Einstein equation (2). Approximate values of 12.4×10^{-6} cm^2 s^{-1} for D and 6.9×10^{-6} for D_{eff} under

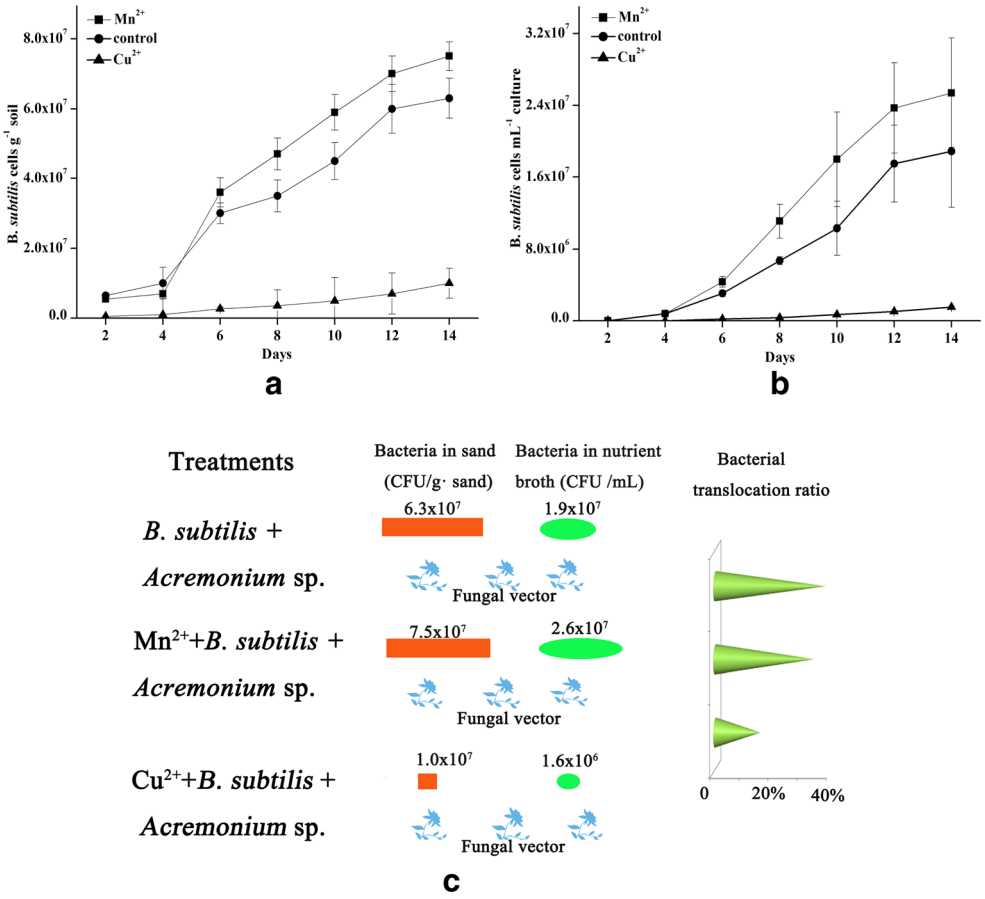

Fig. 4 The influence of heavy metals on the bacterial mobilization along fungal hyphae. The bacterial number in fluoranthene-contaminated sands (**a**) and associated nutrient broth (**b**) in a heterogeneous column system in the presence of Mn^{2+} and Cu^{2+} at 5 mmol/kg·sand. **c** The illustration of the influence of heavy metals on the bacterial mobilization along fungal hyphae

supplementation of Mn^{2+} were obtained, similar with that in the control (Table 2). In the case of Cu^{2+} supplementation, approximate values of 8.5×10^{-6} cm^2 s^{-1} for D and 4.7×10^{-6} for D_{eff} were obtained, which were lower than that in control, representing decreases of 46.8 and 31.9 %, respectively, relative to the control, which was in the range of typical bulk random motility diffusion coefficients as derived from individual cell and population-scale assays in stopped flow diffusion chambers (Lewus and Ford 2001). The bacteria were observed to be present in the mineral medium at the bottom of the apparatus after 72 h in the case of supplementation of Mn^{2+}, which was the same as in the control. However, it took 96 h for the bacteria to reach the medium in the presence of Cu^{2+}, representing an increase of 33.3 % compared with that in the control. These results indicate that the addition of Cu^{2+} in sand imposes a negative impact on the motility diffusion of bacteria in soil or sand, porous media, while the presence of Mn^{2+} has a positive effect on bacterial diffusion, suggesting the influence of HMs on bacterial diffusion is dependent on HMs species.

Discussion

Bioremediation of PAH-polluted sand presented herein with HM supplementation have shown different fluoranthene removal efficiencies using mixed fungal–bacterial cultures, and this can be ascribed to the different effects of individual

Table 1 Number of both bacterial and fungal colony forming units (CFU) detected in a heterogeneous column system inoculated with a mixture of *Acremonium* sp. *and B. subtilis* after 14 days

Experiments	Bacteria detected in contaminated sand (CFU/g sand)	Bacteria detected in mineral nutrient broth (CFU/mL)	Fungal growth in sand (CFU/g·sand)
B. subtilis + *Acremonium* sp.	6.3×10^7	1.9×10^7	3.9×10^5
B. subtilis + *Acremonium* sp. + Mn^{2+}	7.5×10^7	2.5×10^7	4.0×10^5
B. subtilis + *Acremonium* sp. + Cu^{2+}	1×10^7	1.6×10^6	3.8×10^5

CFU is reported in both fluoranthene-contaminated sands in the presence of 5 mmol/kg·sand of either Mn^{2+} or Cu^{2+}, and nutrient medium located below the contaminated sands

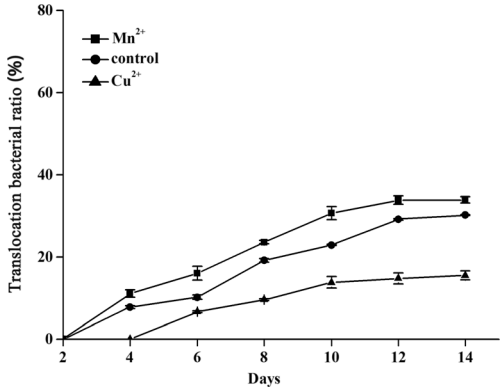

Fig. 5 Bacteria translocation ratio representing the fungal-mediated translocation of bacteria from fluoranthene-contaminated sands in the presence of Mn^{2+} or Cu^{2+} at 5 mmol/kg·sand or a metal-free control

HMs on bacterial growth, translocation ratio along fungal hyphae and bacterial diffusion during bioremediation as they have been confirmed to be related to bioremediation efficiency (Knudsen et al. 2013). Two HMs used in this study have been confirmed to be the representative of individual positive and negative effects on PAHs bioremediation (Ma et al. 2014). Therefore, the work described in this study demonstrated that the presence of different HMs impose different effects on fungal-bacterial synergism in PAH degradation and which exerts its influence on the fungal-bacterial synergism through mobilization via fungal transport vectors, depending on metal species. Further elucidation of the mechanisms of the influence of HMs on fungal-bacterial synergistic PAH degradation among fungi and bacteria with resistance to these metals could greatly improve our understanding of the interaction and synergistic effect between fungi and bacteria in actual polluted environments, and would be helpful to develop an efficient bioaugmentation strategy for such organic waste removal co-polluted with HMs.

In well-defined laboratory experiments presented here, it has indeed been confirmed that fungal hyphae can act as vectors for bacterial mobilization in sand, achieving better substrate bioavailability and thereby more enhanced biodegradation removal compared to that of individual fungus and bacterium, as reported previously (Kohlmeier et al. 2005). More enhanced biodegradation of the particle-associated fluoranthene was found only in the co-presence of both fungal mycelia and bacteria relative to individual fungal and bacterial inoculations, hence demonstrating that the fungus in the consortium also contributes to the enhanced biodegradation through its metabolic synergistic effect. Therefore, more efficient degradation should not be entirely ascribed to the physical vectors of the fungal hyphae acting for bacterial transport, since the PAH-degrading fungus also contributes to the degradation process in spatially heterogeneous systems through its metabolic ability. That is, the synergistic relationships in metabolism between the fungus and the bacterium in a consortium may also exist because the fungi may transform PAHs to metabolites which are more easily degraded by the bacteria or even entirely degrade PAHs, representing possible cooperative catabolism between fungus and bacterium along with the ability of fungal hyphae to act as transport vectors for the bacteria and help in biodegradation, as described previously (Banitz et al. 2013; Knudsen et al. 2013). In the work reported here, more efficient degradation through fungal-facilitated bacterial dispersion was first confirmed and demonstrated for fungal–bacterial consortia both with PAH-degrading ability, which is more consistent with actual environments than previous reports in which the fungus-facilitated mobilization has been mostly demonstrated by common soil fungi (Ellegaard-Jensen et al. 2014; Furuno et al. 2010; Knudsen et al. 2013; Kohlmeier et al. 2005). Thus, this work hereby provides additional experimental and theoretical understanding of microbial interactions within constructed consortia during PAH bioremediation that reflect real-world conditions in contaminated sites.

Table 2 Bacterial diffusion coefficient in the presence of heavy metals (HMs)

Diffusion	Mn^{2+}	Cu^{2+}	Control
D_{eff} ($cm^2\ s^{-1}$)	6.9×10^{-6}	4.7×10^{-6}	6.9×10^{-6}
D ($cm^2\ s^{-1}$)	12.4×10^{-6}	8.5×10^{-6}	12.4×10^{-6}

D the bulk random motility diffusion coefficient, D_{eff} the effective one-dimensional diffusion coefficient

It has been observed that the morphological changes are very common among all groups of fungi cultivated in the presence of some HMs such as fungus *Paxillus involutus* (Darlington and Rauser, 1988). The results presented here showed that no significant difference in the fungal cell weight was observed with the supplementation of individual Mn^{2+} and Cu^{2+} and in the control and there was difference in the ability of fungal hyphae under the respective supplementation of HMs in the system. And this difference therefore could be ascribed to the fungal morphological changes resulting from the presence of different HMs. The faster bacterial diffusion in the presence of Mn^{2+} and in the control relative to that of Cu^{2+} may be explained either by a higher bulk motility of this bacterium and/or by enhanced motility diffusion in the presence of Mn^{2+}, which is consistent with the uni-directional motility of *Escherichia coli* in a previous report (Liu and Papadopoulos 1995). Significantly, based on the results presented here, the presence of HMs imposed different impacts on the functions of fungal hyphae as transport vectors for bacterial mobilization and caused differences in translocation ratio along fungal hyphae in sand, as well as on bacterial growth, depending on the metal species. In addition, it should be noted that models for the bacterial movement in porous media in this study can be applied only with caution to the movement in water films around fungal hyphae as limited as

in capillaries (Berg and Turner 1990), and the presence of HMs may affect the solution chemistry of the water film around hyphae, further imposing effects on the bacterial diffusion in this study.

Experimental and theoretical studies have indicated the coupled effects of chemical conditions and pore space geometry on bacteria transport in porous media (Ford and Harvey 2007) and that significant cell retention in porous media was strongly dependent on the ionic strength (*I*) (Torkzaban et al. 2008). However, as *I* was highly similar between metal treatments in this study, the longer emerging time of bacteria observed in the presence of Cu^{2+} (96 h), in comparison to that under supplementation with Mn^{2+} (72 h), may be explained by the differences between HM species rather than *I*. It was speculated that the different response of bacterial diffusion to the presence of Cu^{2+} and Mn^{2+} resulted in a different structure of porous media, as indicated previously (Ford and Harvey 2007), indicating individual bacterial diffusion behavior. Hence, bacterial diffusion in porous media was a process that strongly depends on the HM species, which is similar to a previous report in which the bacterial retention was thought to be a coupled process that strongly depends on solution chemistry, pore structure, and system hydrodynamics (Torkzaban et al. 2008). In addition, the observed difference in bacterial diffusion may be the conditional dispersal caused by the many factors including changed mycelia or HM stress. However, the bacterial movement was still the flagella-driven swimming, as the "highway" for bacterium provided by fungal mycelia was the presence of water films on fungal hyphae reported previously (Kohlmeier et al. 2005).

Soil bioremediation may be achieved by bioaugmentation; however, this technology is faced with a number of challenges with regard to the presence of HMs, which often cause toxicity to microbial growth and limit degradative ability. Fungal-bacterial consortia for degradation of PAHs have been created with varying degrees of success, showing either no positive effect or an enhanced PAH degradation (Arun and Eyini 2011; Boersma et al. 2010; Machin-Ramirez et al. 2010). Reports concerning the influence of the HMs on the bioaugmentation with consortia including both fungal and bacterial degraders are scarce and to our knowledge, our findings concerning the influence of HMs on the fungal-bacterial synergism in PAH degradation have not been previously reported. The present study shows that the enhanced fungal-bacterial synergism in PAH degradation described here is affected by the presence of HMs coexisting with PAHs, and this depends on the HM species. In particular, the influence of HMs on bacterial diffusion and the translocation ability of fungal hyphae have been clarified. Elucidating the mechanisms through which HMs influence fungal-bacterial synergistic degradation of PAHs could greatly improve our understanding of the interactions and synergistic bioremediation between fungi and bacteria in actual polluted environments, and be helpful in proposing new efficient bioaugmentation strategies for PAHs. The results presented here suggest that the co-inoculation of adapted fungi and bacteria with HM resistance and PAH-degrading ability is a promising bioaugmentation strategy to improve soil bioremediation of soil polluted both with HMs and PAHs in situ or ex situ.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

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