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ORIGINAL ARTICLE

Cyclodextrin-enhanced in situ bioremediation of polyaromatic hydrocarbons-contaminated soils and plant uptake

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Abstract In situ bioremediation of polycyclic aromatic hydrocarbons (PAH) polluted soils can be improved by the augmentation of degrading microbial populations and by the increase of hydrocarbon bioavailability. β-cyclodextrin (β-CD) significantly accelerate the induction of hydrocarbon biodegradation, but it is not still clear its effectiveness during final, slower stages of degradation. Moreover, it is yet not known if the PAH uptake from plants is influenced by the presence of CD. A field study was carried out by creating two plots (A and B). Diesel fuel was spread on the surface, and on plot B a commercial microbial consortium and β-CD were spread. Soybean was seeded in both plots. Soil samples were withdrawn every 10 cm layers from 0 to 60 cm depth, before fuel spreading, immediately after seeding and after soya harvesting. Chemical and microbial analyses were carried out throughout the process to characterize the soil and to determine residual PAHs. Soybean seeds were analyzed for PAH content. It was observed that β-CD induced a significant increase of PAH degradation rate. The microbial inoculum did not improve the degradation; biodegradation activity was strong in superficial layers, and some PAH leaching was observed, that was reduced by CD. The analysis of PAH in soyabeans revealed that an uptake of hydrocarbons occurred, and that it was more significant in plot B. This suggests that the β-CD-enhanced bioremediation process can further be improved by phytoremediation, that could also allow to simultaneously reach an additional profit from a non-food yield for biofuel production.

Keywords β-Cyclodextrin · Bioavailability · Bioremediation · Phytoextraction · Phytostimulation · Polycyclic aromatic hydrocarbons · Soil

Introduction

Hydrocarbons (HC) can be degraded in soil by heterotrophic microorganisms that use them as energy and carbon sources [1, 2]. In cases of accidental HC pollution of soil, a decrease of HC is observed due to natural attenuation, but the degradation of residual HC can become very slow when the more recalcitrant molecules prevail. The degradative activity of soil microorganisms can be improved by the association to plant roots due to phytostimulation, and mycorrhizal fungi can increase the rhizospheric volume where the microorganisms are active improving the efficiency of plant/soil interaction [3, 4]. Nowadays it is not still well known if the inoculation of non-autoctonous degradative microorganisms can accelerate the in situ remediation processes.

Bioavailability is one main factor that influences the extent of biodegradation, as HC are very poorly soluble in the aqueous phase where microorganisms are active [5–8]. HC bioavailability is increased by CDs, that form water-soluble inclusion complexes [9–11]. It has already been demonstrated that β-CD accelerates degradation kinetics of HC both in aqueous and solid phase [12–14]. The β-CD

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effectiveness in in situ bioremediation has also been tested [15]; β-CD significantly accelerates the induction of hydrocarbon biodegradation and improve the degradation rate and extent. However, it is not clear its effectiveness during final, slower stages of degradation, when recalcitrant molecules prevail.

A higher risk of groundwater pollution could be supposed as a consequence of increased HC water solubility, due to greater leaching through the soil profile [16]. The mobility of HC in soil is mainly influenced by organic matter [17, 18]. Various fractions forming the dissolved organic matter (DOM) interact differently with HC [19–21]: humic acids retain HC in the first layers of soil, favouring adsorption by a mechanism of hydrophobic retention, while fulvic acids cause an increased mobility, due to their ability to bind HC and increase their solubility in water; CD effect is yet not known.

Finally, it is yet not known if the polycyclic aromatic hydrocarbons (PAH) uptake from plants is influenced by the presence of CDs; in fact, the uptake usually happens in leaves, while the root uptake and translocation are generally considered improbable, because of their very low water-solubility [3, 4].

In the present work several factors were investigated in order to accelerate and improve the in situ bioremediation of a PAH polluted soil: the degradative activity of indigenous soil microbial populations compared to that due to the addition of a commercial rhizospheric microbial consortium; the effect of the later addition of β-CD on PAH degradation, on heterothrophic microbial population dynamics and on PAH mobility in soil; the interaction among phytostimulation and CDs addition. Moreover, we wanted to evaluate the possibility to further improve soil bioremediation by inducing with β-CD the phytoextraction of PAH. By this way, the bioremediation of the site could also allow an additional profit from a non-food yield, as the plant lipid fractions could be extracted and used as biofuel.

Experimental

Experimental plots

The study was carried out by creating two plots (60 m^2 and 80 cm deep), named A and B, that were impermeabilized with polyethylene sheets and filled with agrarian soil. At time 0, diesel fuel was spread on the surface (250 g/m^2), and 50 g/m^2 urea were added to provide the nitrogen necessary to allow the microbial growth. In plot B a commercial microbial consortium was spreaded (200 g/m^2) containing rhizospheric microorganisms (*Glomus caledonium* GM24, *Glomus intraradices* GG31, *Glomus coronatum* GU53, *Pseudomonas fluorescens* PA28, *Pseudomonas borealis* PA29, *Bacillus subtilis* BA41). Soybean (*Glycine max*), chosen due to its high lipid content, was seeded in both plots. β-CD (170 g/m^2) was added 42 days later in plot B. The schedule of plots management is reported in Table 1.

Soil samples were taken at 10 cm interval to 60 cm immediately after seeding (time 1) and after soya harvesting (time 2). Chemical analysis were carried out to determine pH, cation exchange capacity (CEC), total carbon, nitrogen and phosphorus and residual total PAHs. Microbiological analysis was also carried out to determine total heterothrophic microorganisms and PAH-degrading microorganisms. The soybean seeds were analyzed to determine the residual PAH. Soil and seed samples were also analysed from a close, not-polluted soybean field.

Soil analysis

Soil samples were air-dried, mildly reduced to particles and sieved at 2 mm and 0.5 mm with plastic sieves to avoid any contamination. Soil chemical properties were determined following ISO methods: the pH was determined in $CaCl_2$ solution, 1:5 soil/solution ratio (ISO 10390, 1994), organic carbon and total nitrogen by CE Instruments NA 2100 elemental analyser (ISO 10694, 1995), and CEC with $BaCl_2$ at pH 8.1 (ISO 13536, 1995). The P-absorbed was determined following the P-Olsen analysis (MRP), reported by I.S.S.S. methods.

Total heterothrophic microorganisms were determined as Colony Forming Units (CFU)/g of soil by extracting microorganisms in saline (30 min, 250 rpm) and plating on 3M™ Petrifilm™ ''Aerobic Count Plate'' (ACP) and in 3M™ Petrifilm™ ''Yeast and Mould Count Plate'' (YMCP). Total PAH degrading microorganisms (PAH DM) were determined by plating on selective growth medium (BHA with diesel fuel as the sole energy and carbon source). The plates were incubated at 30 °C for 7–10 days.

Table 1 Schedule of experimental plots management

	Plot A	Plot B
Early May—Time 0	Diesel fuel pollution	Diesel fuel pollution Microbial consortium addition
Mid May	Soya seeding	Soya seeding
End June—Time 1	1st sampling	1st sampling β-CD addition
Early October	Soya harvesting	Soya harvesting
End October—Time 2	2nd sampling	2nd sampling

PAH analysis

PAH were extracted from soil and from soybean seeds in hexane following the EPA3550C method. Quantitative analysis was performed by a Jasco LC-2000 Plus HPLC System equipped with a UV/Vis UV-2075 Plus detector according to EPA 610 method and expressed as total PAH (mg/kg).

Results

Soil chemical analysis results are shown in Table 2. The soil parameters were homogeneous through the soil profile, as expected due to soil mixing occurred during plot filling. Soils presented an homogenous texture, with a high sand content. The pH was around neutrality, this parameter ranging from 7.0 to 7.5. CEC was similar throughout the soil profile and in the two plots, being on average equal to 16 cmol/kg. The C/N ratio was equilibrated and the high P Olsen content (84 mg/kg on average) reflected a probable use of fertilizers in the past.

The microbial populations of the different soil layers at time 1 (june) and 2 (october) in plot A and B are reported in Fig. 1. In all samples at time 1 the microorganisms were much more abundant respect the non-polluted soil sampled from the near field, where 1×10^6 (ACP), 9×10^2 (YMCP) and 0 (PAH DM) CFU/g were detected. Microorganisms were in general concentrated in superficial layers of soil (0–20 cm), with the exception of yeasts and moulds, that shown a peculiar distribution through the soil profiles but also an always low concentration; in plot B microorganisms were significantly more concentrated than in plot A. In the 0–10 cm layer an increase of total microorganisms (ACP and YMCP) was observed from time 1 to 2, while PAH degrading microorganisms decreased: in plot B the decrease was about 75%, reaching a value very near to plot A. In 10–20 cm layers total microorganisms decreased from time 1 to 2, in particular in plot B, where an increase of them was observed in deepest soil layers. Yeasts and mould strongly increased in plot B, except in 50–60 layer.

Table 2 Soil chemical characterisation at time 0. Plot A and Plot B, time 0: see Table 1

	Depth cm	pH in $CaCl_2$	C.E.C. cmol(-)/kg	C/N	P mg/kg
Plot A	0–10	7.4	14	11	68
	10–20	7.5	15	11	75
	20–30	7.3	15	11	69
	30–40	7.0	17	10	103
	40–50	7.3	17	10	106
	50–60	7.1	18	11	96
Plot B	0–10	7.3	16	10	92
	10–20	7.0	16	10	108
	20–30	7.1	16	10	107
	30–40	7.0	16	10	92
	40–50	7.0	18	10	110
	50–60	7.1	17	10	100
Average		7.2	16	10	94
SD		0.2	1.4	0.6	15.2
C.V.%		2.3	9.2	5.4	16.2

PAH degradation calculated on the basis of the average residual concentration (mg/kg) in plot A and B is reported in Fig. 2. The decrease from time 0 to 1 was very strong in both plots, but the residual concentration was significantly higher in plot B (25.7 ± 4.6 mg/kg) than in A (6.2 ± 3.8 mg/kg). From time 1 to 2 the % decrease was higher in plot B (60%) than in A (32%).

The distribution of residual PAH along the profile was different (Table 3): at time 1, in plot A 78% was in 0–10 cm layer and some residue was present also at 40–50 cm (3%) and 50–60 cm (7%), while in plot B 71% was present at 0–10 cm and no residue was found in deepest layers. At time 2, in plot A the highest residual concentration was found in deepest layers: 43% at 40–50 cm and 19% at 50–60 cm, while at 0–10 cm it was 38%; in plot B the highest residual concentration was at 30–40 cm (48%), while it was 20% at 0–10 cm and 18% at 20–30 cm.

The analysis of PAH extracted from soybean seeds revealed the presence of different amount of PAH in seeds coming from the two plots: the PAH content was quantitatively and qualitatively higher in seeds from plot B, where three compounds were observed that were absent in plot A (unshown data), and a PAH concentration of 0.018 ± 0.001 mg/g of seeds was detected.

Discussion

The addition of diesel fuel to soil induced a rapid and significant microbial growth, with the appearance of PAH degrading microorganisms in both plots. The microbial consortium added in plot B produced a general and significant increase of all the checked rhizospheric microbial populations (Fig. 1), but this wasn't effective on the PAH degradation improvement: in fact, the decrease of PAH observed at time 1 in plot A was higher than in B (Fig. 2), probably due to the lag-phase of non-indigenous microorganisms inoculated in soil. This means that increasing microorganisms concentration is not useful to accelerate degradation, and confirms that the main limiting factor is the bioavailability; in fact, at time 2, following the (-cyclodextrin addition, the degradation rate was accelerated in plot B (Fig. 2). The decrease of PAH degrading microorganisms from time 1 to 2 is explained by the fact

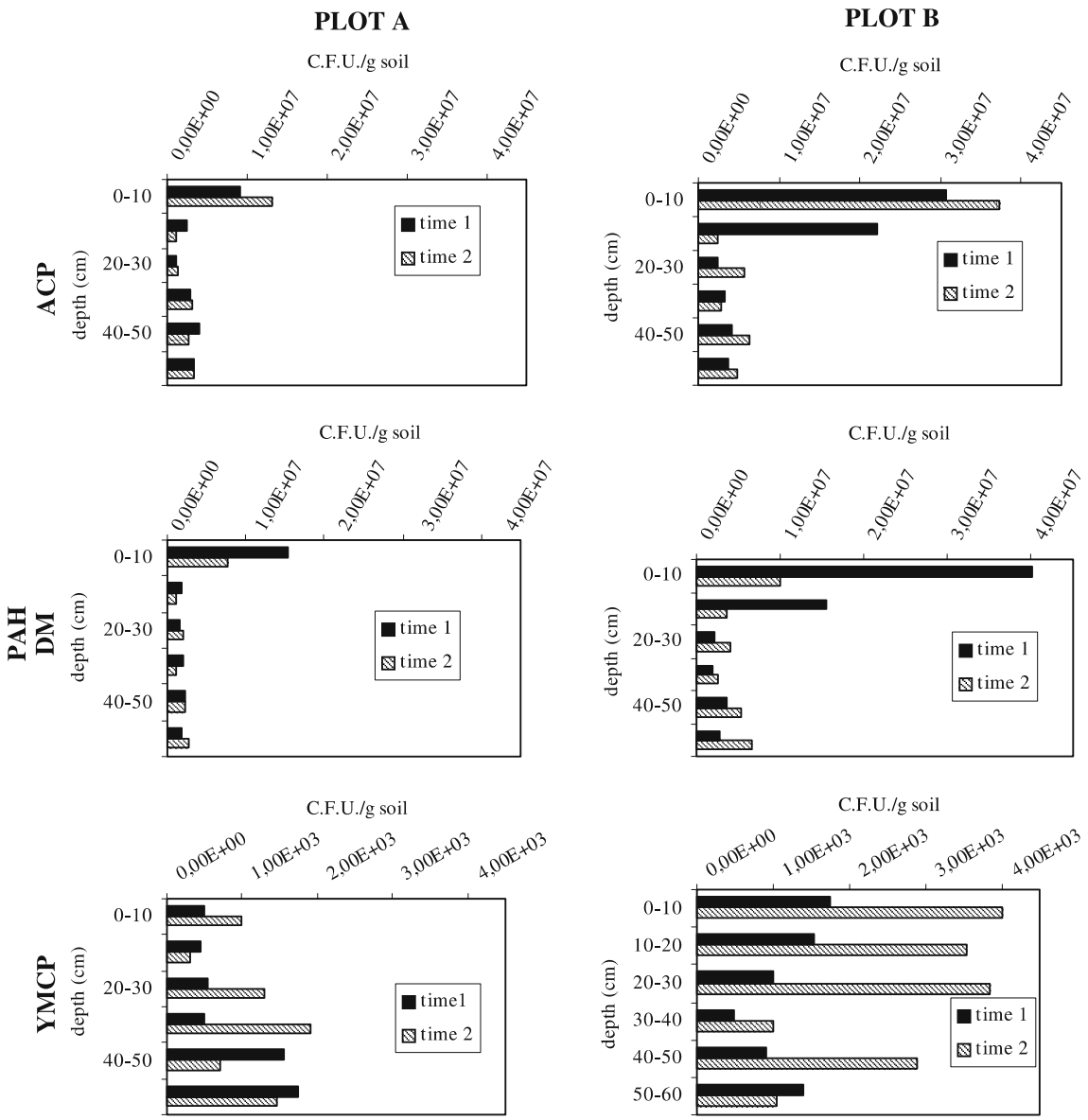

Fig. 1 Concentration of microorganisms in soil (C.F.U./g). ACP = - Total heterothrophic microorganism grown on Petrifilm™ ''Aerobic Count Plate; PAH DM = PAH degrading microorganism grown on selective medium (Bushnell Haas Agar with fuel addition). YMCP = Yeast and Mold grown on Petrifilm™ ''Yeast and Mold Count Plate''.Time 1 and time 2, plot A and plot B: See Table 1

that PAH as energy and carbon sources were nearly disappeared (Fig. 2), while the rhizospheric environment created by the association of plant roots and microorganisms gave rise to a general increase of the other microbial populations (Fig. 1). The dynamics of microbial populations at different depth were influenced by several factors: air availability, presence of plant roots, PAH concentration, but anyway the main growth is concentrated in superficial layers.

The distribution of residual PAH along the soil profile (Table 3) showed that a leaching of PAH happened and that it was significantly higher in plot A. That means that β-CD can give a protection from the PAH leaching, showing a behaviour similar to humic acids, that retain HC in superficial layers [16].

The addition of β-CD was made 42 days after the pollution: at this time, the most recalcitrant molecules were residual, and β-CD shown to be effective improving their microbial degradation rate, then their bioavailability (Fig. 2): in fact, even if the final residual concentration remained lower in plot A, the degradation rate was nearly 10-fold higher in plot B than in A (0.13 $\mu g/kg^{-1}/day^{-1}$ agains 0.016 $\mu g/kg^{-1}/day^{-1}$). Although it was supposed that the late addition could be a way to prevent PAH leaching, it

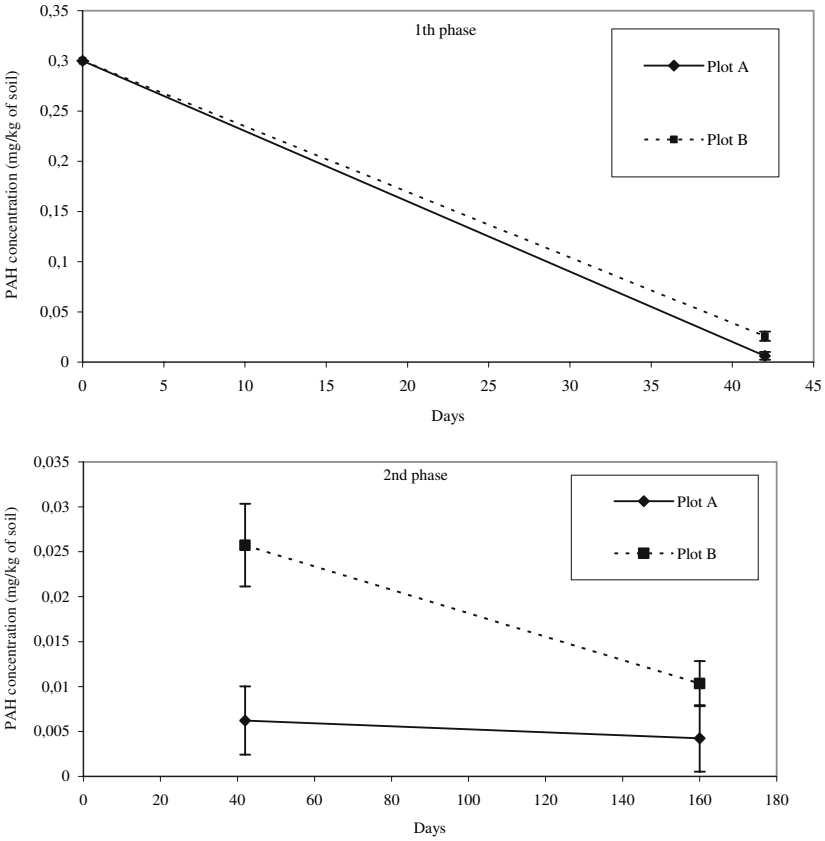

Fig. 2 PAH decrease in soil. Concentration expressed as mg/g of soil and calculated as the average from analysis of six soil layers. 1st phase: from time 0 to 1; 2nd phase: from time 1 to 2 (see Table 1)

Table 3 Residual PAH distribution along the soil profiles (%). Time 1 and time 2, plot A and plot B: see Table 1

Depth (cm)	Plot A		Plot B	
	Time 1	Time 2	Time 1	Time 2
0–10	78	38	71	20
10–20	9	0	9	18
20–30	0	0	19	8
30–40	3	0	1	48
40–50	3	43	0	2
50–60	7	19	0	5

was found that β-CD did not increase the PAH leaching, but that on the contrary it reduced this risk.

Finally, the analysis of residual PAH in soybean seeds revealed that traces of PAH were present from both plots: this suggests that a HC root uptake and xylematic translocation occurred. Then bioremediation of PAH contaminated soils is necessary before their agricultural use, to prevent the health risk of the presence of PAH in foods, because the uptake happened in roots. PAH content of seeds was higher and qualitatively different in plot B: this means that the higher water-solubility induced by β-CD also increased PAH root uptake and xylematic translocation. However, an additional effect of symbiotic mycorrhizal fungi can also be hypothesized on increased plant uptake, that will be further investigated in next work. Anyway, in situ PAH bioremediation is improved by associating β-CD to phytoremediation, also allowing to reach an additional profit from a non-food yield for biofuel production.

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References

1. Cookson, J.T.: Optimizing microbial transformation of hazardous chemicals. In: Cookson, J.T. (ed.) Bioremediation Engineers, McGraw-Hill, Inc., USA pp. 95–164. (1995)
2. Holliger, C., Gaspard, S., Gold, G., Heijman, C., Schumacher, W., Schwarzenbach, R.P., Vazquez, F.: Contaminated environments in the subsurface and bioremediation: organic contaminants. FEMS Microbiol. Rev. **20**, 517–523 (1997)
3. Henner, P., Schiavon, M., Morel, J.L., Lichtfouse, E.: Polycyclic aromatic hydrocarbon (PAH) occurrence and remediation methods. Analusis **25**, 56–59 (1997)
4. Huang, X.D., El-Alawi, Y., Penrose, D.M., Glick, B.R., Greenberg, B.M.: A multi-process phytoremediation system for removal of polycyclic aromatic hydrocarbons from contaminated soils. Environ. Pollut. **130**, 465–476 (2004)

5. Barkay, T., Navon-Venezia, S., Ron, E.Z., Rosenberg, E.: Enhancement of solubilization and biodegradation of polyaromatic hydrocarbons by the bioemulsifier alasan. Appl. Environ. Microbiol. **65**, 2697–2702 (1999)
6. De Jonge, H., Freijer, J.L., Verstraten, J.M., Westerveld, J., Van der Wielen, F.W.M.: Relation between bioavailability and fuel oil hydrocarbon composition in contaminated soils. Environ. Sci. Technol. **31**, 771–775 (1997)
7. Tabak, H.H., Goving, R.: Bioavailability and biodegradation kinetics protocol for organic pollutant compounds to achieve environmentally acceptable endpoints during bioremediation. Ann. N.Y. Acad. Sci. **829**, 36–61 (1997)
8. Szejtli, J.: Cyclodextrin Technology, 393 p. Kluwer Academic Publishers, Dordrecht (1988)
9. Cerniglia, C.E., Heitkamp M.A.: Microbial degradation of polycyclic aromatic hydrocarbons in the aquatic environment. In: Varanasi U. (ed.) Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment, CRC Press, Boca Raton, FL, USA pp. 41–68 (1989)
10. Olah, J., Cserhati, T., Szejtly, J.: Beta-cyclodextrin enhanced bilogical detoxification of industrial wastewater. Water Res. 22, 1345–1352 (1988)
11. Wang, J.M., Marlowe, E.M., Miller-Maier, R.M., Brusseau, M.L.: Cyclodextrin-enhanced biodegradation of phenanthrene. Environ. Sci. Technol. **32**, 1907–1912 (1998)
12. Bardi, L., Mattei, A., Steffan, S., Marzona, M.: Hydrocarbon degradation by a soil microbial population with β-cyclodextrin as surfactant to enhance bioavailability. Enzyme. Microb. Technol. **27**, 709–713 (2000)
13. Bardi, L., Steffan, S., Marzona, M.: Biorisanamento in situ di suoli inquinati da idrocarburi con ciclodestrine come coadiuvanti per incrementare la biodisponibilità. Bollettino Società Italiana di Scienza del Suolo **51**, 259–268 (2002)
14. Steffan, S., Bardi, L., Marzona, M.: Biodegradation of hydrocarbons in polluted soils using β-cyclodextrin as a coadjuvant. Biol. J. Armenia, Special Issue: Cyclodextrins, 218–225 (2001)
15. Bardi, L., Ricci, R., Marzona, M.: In situ bioremediation of a hydrocarbon polluted site with cyclodextrin as a surfactant. WASP: Focus **3**, 15–23 (2003)
16. Petruzzelli, L., Celi, L., Cignetti, A., Ajmone Marsan, F.: Influence of soil organic matter on the leaching of polycyclic aromatic hydrocarbons in soil. J. Environ. Sci. Health **37**, 187–199 (2002)
17. Raber, B. Kogel-Knabner, I.: Influence of origin and properties of dissolved organic matter on the partition of PAH. Eur. J. Soil Sci. **48**, 443–455 (1997)
18. Chiou, C.T., Malcom, R.L., Brinton, T.I., Kile, D.E.: Water solubility enhancement of some organic pollutants and particles by dissolved humic and fulvic acids. Environ. Sci. Technol. **20**, 502–508 (1986)
19. Deschauer, H., Hartmann, R., Kogel-Knaber, I., Zech, W.: The influence of dissolved organic matter on the transport of polycyclic aromatic hydrocarbons (PAHs) in a forest soil under *Pinus Silvestris.* In: Senesi, N., Miano, T.M. (eds.) Humic Substances in the Global Environment and Implications on Human Health, pp. 1063–1069. Elsevier Science, New York, USA (1994)
20. Totsche, K.U., Danzer, J., Kogel-Knaber, I.J.: DOM-enhanced retention of polycyclic aromatic hydrocarbons in soil. J. Environ. Qual. **26**, 1090–1100 (1997)
21. Raber, B., Kogel-Knaber, I., Stein, C., Klem, D.: Partitioning of polycyclic aromatic hydrocarbons to dissolved organic matter from different soils. Chemosphere **36**, 79–97 (1998)