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The effect of humic acids on biodegradation of polycyclic aromatic hydrocarbons depends on the exposure regime

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ABSTRACT

Binding of polycyclic aromatic hydrocarbons (PAHs) to dissolved organic matter (DOM) can reduce the freely dissolved concentration, increase apparent solubility or enhance diffusive mass transfer. To study the effects of DOM on biodegradation, we used phenanthrene and pyrene as model PAHs, soil humic acids as model DOM and a soil *Mycobacterium* strain as a representative degrader organism. Humic acids enhanced the biodegradation of pyrene when present as solid crystals but not when initially dissolved or provided by partitioning from a polymer. Synchronous fluorescence spectrophotometry, scintillation counting and a microscale diffusion technique were applied in order to determine the kinetics of dissolution and diffusive mass transfer of pyrene. We suggest that humic acids can enhance or inhibit biodegradation as a result of the balance of two opposite effects, namely, solubilization of the chemicals on the one hand and inhibition of cell adhesion to the pollutant source on the other.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants with recognized toxicity and carcinogenicity potentials. Biodegradation is one of the most direct pathways for dissipation of these chemicals in polluted soils and sediments. However, the microbial availability of PAHs is often limited by their low water solubility and their high tendency of sorption to the solid surfaces of these environmental compartments, which causes slow biodegradation rates and longer persistence (Juhasz et al., 2010; Posada-Baquero and Ortega-Calvo, 2011). These representative hydrophobic organic contaminants (HOCs) also tend to associate to dissolved macro-molecules (such as humic acids or HA) present in the porewaters. This association will in some exposure scenarios lead to a decrease in the freely dissolved concentrations and then likely to lower biodegradation rates, because degrading bacterial cells usually take up dissolved PAHs by diffusion from the surrounding aqueous phase (Thomas et al., 1986; Bosma et al., 1997; Alexander, 1999; Wick et al., 2001; Yang et al., 2009). However, in other situations the presence of dissolved organic matter (DOM) can lead to an enhancement in the biodegradation rate of these HOCs (Ortega-Calvo and Saiz-Jimenez, 1998; Haftka et al., 2008; Smith et al., 2009). Indeed, the addition of DOM (such as HA) is currently considered as a valid biostimulation strategy to speed up bioremediation (Plaza et al., 2009; Yang et al., 2011). Various forms of DOM including humic acids can accelerate the biodegradation of PAHs by increasing the apparent solubility and enhancing the diffusive mass transfer of these contaminants, thus promoting their availability to microorganisms (Smith et al., 2009). The molecular form of HA has been described as a micellar microstructure similar to surfactants, which can incorporate dissolved PAHs (Engebretson and Vonwandruszka, 1994). Despite these studies that show the importance of HA in bioremediation, its exact role in the degradation of PAHs by microorganisms remains uncertain. The relative contributions to the biodegradation process by the decreases in freely dissolved concentrations, the enhanced diffusion, and the increases in the total load of pollutant present in the aqueous phase due to enhanced solubilization, still remain unknown. The clarification of these effects can be relevant for predicting the effects of DOM on biodegradation of PAHs in natural and engineered environments.

The present study puts forth a new combination of two analytical techniques, synchronous fluorescence spectrophotometry and liquid scintillation counting, to approach this question, by determining the freely dissolved concentration (C_{free}) and the total concentration in the aqueous phase (C_{total}, where $C_{total} = C_{free} + C_{bound\text{-}HA}$) of pyrene in different exposure regimes. The combination of these techniques allowed the determination of

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the distribution between bound and unbound pyrene in aqueous solutions containing HA, while avoiding any phase separation steps. The approach also allowed establishing the dynamics of C_{free} during biodegradation, in the presence and absence of HA. A passive dosing technique, adapted to biodegradation procedures, was also applied to change the exposure regime, as compared with the crystalline PAH. This approach uses a preloaded silicone polymer for continuously supplying the contaminant (Smith et al., 2010), which causes a different exposure regime of the degrading cells because the contaminant is introduced by phase partitioning and without any addition of co-solvents or microcrystals. The usefulness of passive dosing within studies focused on biodegradation processes was recently shown by Smith et al. (Smith et al., 2012), who used a miniaturized set-up with silicone O-rings (as dosing polymer), and opened new possibilities for studying biotransformation of HOCs at environmentally relevant scenarios. The main goal of our study was to investigate whether the enhancing effect of HA on PAH biodegradation depends on the different dosing regimes in which the microorganisms are exposed to PAHs.

2. Experimental section

2.1. Chemicals

Non-labeled PAHs, pyrene (purity 98%) and phenanthrene (98%) were purchased from Aldrich Chemical Co. Phenanthrene-9-^{14}C (13.1 mCi/mmol, radiochemical purity > 98%) was supplied by Sigma Chemical Co and pyrene-4,5,9,10-^{14}C (58.8 mCi/mmol, radiochemical purity of 99.0%) by Campro Scientific GmbH. The solubility in water of phenanthrene and pyrene is 1.18 μg/mL and 0.13 μg/mL, respectively.

2.2. Bacterium, media and cultivation

The bacterium used in this study, *Mycobacterium gilvum* VM552, originated from a PAH-polluted soil and is capable to use phenanthrene and pyrene as its sole source of carbon and energy. The bacterium was cultured with phenanthrene and prepared for biodegradation experiments as described elsewhere (Tejeda-Agredano et al., 2011).

2.3. Humic acids

Humic acids (HA) originated from a soil located in the National Park of Doñana, near to Laguna de Santa Olalla (Huelva, Spain). The detailed composition of this soil humic fraction is described elsewhere (Lahlou et al., 2000). A stock solution of HA (1 mg/mL) was prepared in sodium hydroxide (0.1 M), and the pH was adjusted to pH = 6 with HCl (Ortega-Calvo and Saiz-Jimenez, 1998). The stock solution was diluted with an inorganic salt solution (MM, pH 5.7) to achieve the desired concentration of HA (10 and 100 μg/mL of total organic carbon or TOC). The MM solution had the following composition: 900 mg/L KH_2PO_4, 100 mg/L K_2HPO_4, 80 mg/L $CaCl_2$, 100 mg/L NH_4NO_3, 100 mg/L $MgSO_4{\cdot}7H_2O$, 2 μg/L $Na_2B_4O_7{\cdot}10H_2O$, 2 μg/L $MnSO_4{\cdot}H_2O$, 2 μg/L $CuSO_4{\cdot}5H_2O$, 1.4 μg/L $Na_2MoO_4{\cdot}2H_2O$, 2 μg/L $ZnSO_4{\cdot}H_2O$, and 10 μg/L $FeCl_3{\cdot}6H_2O$. These HA concentrations were environmentally representative because DOC concentrations in soil pore water are usually not higher than 100 μg/mL (Deflandre and Gagne, 2001), whereas the concentration range for most natural freshwaters is between 0.5 and 50 μg/mL of DOC (Bittner et al., 2011). TOC estimations were carried out at IRNAS-CSIC with a TOC Analyzer (model TOC-V CPH, Shimadzu, Japan). The surface tension of HA solutions was determined at 25 °C with a TD1 Lauda ring tensiometer and it was not significantly different to MM (60 mN m^{-1}).

2.4. Mineralization of water-dissolved and crystalline PAHs

Experiments with water-dissolved PAHs were performed with 5-mL suspensions of MM containing HA (at 10 or 100 μg/mL), bacteria and the corresponding ^{14}C-PAH at $6{\cdot}10^{-4}$ μg/mL. The ^{14}C-PAH had been dissolved completely in MM before use (3000–4000 dpm per tube). The suspensions contained $2.4{\cdot}10^8$ cells/mL (optical density or OD_{600} = 0.2, 337 μg protein/mL). The suspensions were introduced into 15 mL Pyrex tubes, and a glass rod (0.5 cm in diameter and 2 cm long) was placed inside each tube to assist mixing during incubation. The tubes were closed with Teflon wrapped plugs. Vials (2 mL of capacity) with 1 mL of NaOH (0.5 M) were suspended from the plugs to trap $^{14}CO_2$. The tubes were incubated at 25 °C on an orbital shaker operating at 80 rpm. To measure mineralization, NaOH samples were taken from each vial at predetermined time points and were replaced with fresh NaOH. The samples were mixed with 5 mL of liquid scintillation cocktail (Ultima Gold supplied by Perkin Elmer Spain, S.L.) for quantifying $^{14}CO_2$ by measuring radioactivity in a liquid scintillation counter (Beckman instruments, Inc., Fullerton CA; model LS6500). Maximum mineralization rates were calculated as the slope of the regression lines drawn with the points belonging to the phase of maximum mineralization (Ortega-Calvo and Alexander, 1994). To measure mineralization of crystalline PAHs, known volumes of acetone with ^{14}C-PAH (100 μL, 40,000 dpm per tube) and sufficient unlabeled PAHs to provide the desired final concentration were left to evaporate at the bottom of 15 mL Pyrex tubes. The concentration of PAHs chosen were slightly above their aqueous solubility 1.44 μg/mL for phenanthrene and 0.16 μg/mL for pyrene, or well above their aqueous solubility 5 μg/mL for both compounds. Five milliliters of MM containing HA (10 or 100 μg/mL) and bacteria ($2.4{\cdot}10^8$ cells/mL) were then added. No bacterial growth was expected, according to the cell density and the initial concentration of carbon used in the experiments. Radioactivity was counted as $^{14}CO_2$ dissolved in NaOH traps as the mineralization experiments with dissolved hydrocarbons described above. The statistical study was made with SPSS 18 program, using ANOVA of one factor and Tukey's HSD test.

2.5. Mineralization with passive dosing

The experiments were performed in duplicate 20 mL glass vials that contained 500 ± 5 mg of poly(dimethylsiloxane) (PDMS) fixed on their bottom. The procedure for preparation of these vials and their sterilization are described in the Supplementary material. The resulting PDMS elastomer had a thickness of 1 mm, a total volume of 0.49 mL and a contact area for passive dosing of 4.91 cm^2 (assuming a flat surface). The PDMS was preloaded with 0.1 mL of a solution of pyrene in acetone (250 μg/mL) to give the same amount of pyrene per vial as in the experiments with the crystalline chemical when it was added at a concentration of 5 μg mL^{-1}. The concentration of pyrene in the PDMS was 51 μg/mL. ^{14}C-pyrene was also present in the loading acetone solution to give 30,000–40,000 dpm per vial. The acetone was evaporated completely within 24 h, which resulted in a quantitative transfer of pyrene into the PDMS. Five milliliters of a MM solution with HA (10 μg/mL) and bacteria ($2.4{\cdot}10^8$ cells/mL) were then added. The rest of the procedure was the same as described above for mineralization experiments of water-dissolved and crystalline PAHs.

Mass balances were performed after mineralization experiments by quantification of the ^{14}C activity remaining in the suspensions, and they accounted for 90–110% of the initial radioactivity, what confirmed the absence of significant unaccounted losses.