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Potential use of a self-dying reporter bacterium to determine the bioavailability of aged phenanthrene in soil: Comparison with physicochemical measures

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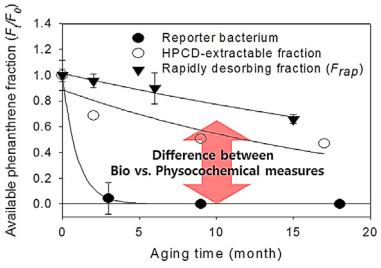
HIGHLIGHTS

- A self-dying reporter bacterium quantified the bioavailable phenanthrene aged in soil.
- A remarkable decrease of bioavailable phenanthrene was observed with the reporter bacterium.
- In aged and organic rich soils, physiochemical measures may overestimate the bioavailability.

GRAPHICAL ABSTRACT



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ABSTRACT

The potential bioavailability of phenanthrene aged in soil was determined by using a self-dying reporter bacterium, and the results were compared to two physicochemical measures, Tenax TA® bead-assisted desorption, and hydroxypropyl-β-cyclodextrin (HPCD) extraction. The reporter bacterium, capable of degrading phenanthrene as a sole carbon and energy source, was genetically reconstructed to die when it degrades phenanthrene. Therefore, population change of the reporter cells can be viewed as the quantification of bioavailable phenanthrene. When Ottawa sand was used as an aging matrix, the amounts of bioavailable phenanthrene (i.e. little gradual decrease) were similar, regardless of aging time, and consistent between the reporter bacterium and the two physicochemical measures. However, decrease in bioavailable phenanthrene with aging was readily evident in sandy loam with organic matter of 11.5%, with all three measures. More importantly, when the reporter bacterium was used, a rapid and significant decrease in the bioavailable fraction from 1.00 to 0.0431 was observed. The extent of decrease in bioavailable fraction was less than 40% in the two physicochemical measures, but was nearly 100% in the reporter bacterium, during the first 3 months of aging. Our results suggest that the phenanthrene fraction available to bacterial degradation, and probably the fraction that really manifests toxicity, may be much smaller than the fractions predicted with the physicochemical measures.

1. Introduction

Hydrophobic organic compounds that persist in soil exhibit declining extractability and bioavailability to microorganisms and other soil organisms with increasing soil contact time, and the term "aging" is applied to the phenomenon [1–3]. During the aging process, the organic compounds partition into the soil organic matter, and/or move into less accessible sites through soil micropores. Since soil organic matter is a heterogeneous hydrophobic microporous medium, hydrophobic molecules become resistant to desorption, and thus less or nonbioavailable [4–7]. Studies using isotope-labeled compounds have demonstrated that this sequestrated fraction of the compounds becomes non-available

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and non-extractable residues [8], and its proportion increases with increasing soil contact time [5–7,9]. The organic matter content and porosity of soil are known to be major determinants of aging [5].

Information on the aging of hydrophobic organic compounds is particularly important, because total concentrations of those compounds detected in soil by vigorous extraction may overestimate the risk to potential receptors, and also provide an inaccurate indication of the cleanup/bioremediation potential. There have been several attempts to determine bioavailability change with aging by biodegradation [1,10,11], mild extraction such as hydroxypropyl-β-cyclodextrin (HPCD) extraction [12–14], desorption kinetic by Tenax TA® bead [15,16], and bioaccumulation assay [17]. Genetically modified microorganisms have also been used to detect environmental contaminants in soil. One approach is to use recombinant bacteria that constitutively emit fluorescence or luminescence. Such reporter bacteria are designed to die, and thus lose signals when they contact with target contaminants, due to the toxicity of the compounds. Shaw et al. [18] used a *lux*-marked bacterium *Burkholderia* sp. RASC, to detect the toxicity of 2,4-dichlorophenol in soil. The detection range was from 10 to 50 mg/L in soil–water extract (soil:water ratio, 1:2.5, w/v). Gu et al. [19] also used recombinant bioluminescent bacterium *E. coli* RFM443 to detect the toxicity of phenanthrene in soil, and the detection range was 0–20 mg/L in soil-extract with the aid of a surfactant. Such a detection system depends on the toxicity response (i.e. cell death) to target contaminants. If the bioreporter has resistance to the contaminant, for instance, it would not die, and thus cannot accurately detect its toxicity. Another strategy is using bioreporters, which generate fluorescent or luminescent signals, when specific genes cloned into the bioreporters are induced by target contaminants. Bioluminescent bacterial biosensor *Pseudomonas putida* TVA8, of which luminescence expression is proportional to induction of the *tod* promoter [20], and a double-tagged fluorescent reporter bacterium *Burkholderia sartisoli* sp. strain RP037-mChe, which emits green fluorescence by phenanthrene flux to the cell [21], have been used to detect BTEX (benzene, toluene, ethylbenzene, xylene) compounds and phenanthrene, respectively. However, the former study involves the extraction of BTEX with water to measure water-extractable soil-bound BTEX, instead of bioavailable BTEX. The latter one used a gel-embedded system to detect phenanthrene diffused through the gel from the soil. It is not likely that either one seems to represent the actual bioavailable fraction of the target compound, because both systems could not reflect the interactions between soil, contaminant and bacteria.

Recently, we constructed a novel reporter bacterium that is designed to die on the initiation of phenanthrene biodegradation. The reporter bacterium successfully quantified the bioavailability of phenanthrene in Ottawa sand, model solids, and sandy loam, but the phenanthrene was all freshly spiked [22,23]. In this study, we tested the reporter bacterium with phenanthrene-aged soils, and easily available fractions of phenanthrene were determined by the bioreporter and two commonly used physicochemical measures (i.e. HPCD extraction and Tenax TA desorption), and the discrepancy in predicting the bioavailability of soil-residing phenanthrene between the methods was discussed.

2. Materials and methods

2.1. Soil preparation and chemicals

Phenanthrene was purchased from Sigma Chemicals Co. (St Louis, MO, USA), and HPCD (hydroxypropyl-β-cyclodextrin) was obtained from Acros Organic (NJ, USA). Tenax TA (60–80 mesh; Sigma) beads were rinsed with acetone and hexane, and dried overnight at 75 °C, before use. The soils used in this study were Ottawa sand (particle diameter, 500–850 μm; surface area, 0.13 m^2/g; Fisher Scientific, PA, USA), and sandy loam (sand 53.3%, silt 27.7%, clay 19.0%), with 11.5% of organic matter and 2.4 m^2/g of surface area. The surface area was determined by N_2-BET analysis (Micrometrics ASAP2420; Micromeritics Instrument Corporation, GA, USA). The sandy loam was air-dried, and passed through a 2-mm sieve. The Ottawa sand and sandy loam were sterilized with 2.5 Mrad of γ-irradiation from a ^{60}Co source.

The spiking procedure for phenanthrene into soil was adapted from the study of Reid et al. [24] and, Kelsey and Alexander [3], with some modifications. One hundred grams of sterile Ottawa sand or sandy loam were added aseptically to a sterile 1-L media bottle, to which 5, 15, 25, and 50 mg of phenanthrene dissolved in 0.5 mL of methylene chloride solution was added, at a concentration of 50, 150, 250, and 500 mg/kg-soil. The same quantity of pure methylene chloride was added to the blank. The soil sample was mixed thoroughly for 2 min, and the solvent was evaporated, by placing the bottle in a fume hood for 10 min. This procedure was repeated, until the bottle contained a total of 500 g of soil sample. The bottle was then put in a hood overnight, to evaporate any residual methylene chloride in soil. Sterile 125-mL serum bottles were filled with phenanthrene-spiked sand or sandy loam without any headspace, and closed with black butyl rubber stoppers and aluminum cap crimping. The resulting amounts of phenanthrene-spiked sand and sandy loam in the serum bottles were 268.7 ± 2.93 g and 149.8 ± 4.5 g, respectively. The bottles were stored at 25 ± 1 °C in the dark, for the aging period of 3, 9, and 18 months.

2.2. Reporter bacterium

A self-dying reporter bacterium, strain S, is the recombinant bacterial species constructed from *Sphingomonas paucimobilis* EPA505 harboring pBBR1PGEF. Detailed descriptions about the reporter bacterium are presented in our previous study [23]. Briefly, strain S contains the phenanthrene-inducible promoter (i.e. the promoter site of *pbhA* gene) fused with *gef* gene (i.e. gene expression fatal) in the pBBR1 MCS-2 vector. Since the *pbhA* promoter initiates phenanthrene biodegradation, strain S can biodegrade phenanthrene, which turns on *gef* gene, a self-killing gene [25]. Consequently, strain S is designed to die on the initiation of phenanthrene biodegradation, and thus its fluorescence is diminished, when stained by using a live/dead cell staining method. Therefore, the signal obtained from the staining decreases, as phenanthrene biodegradation increases. The reporter bacterium was cultured at 30 °C in tryptic soy broth (TSB) or mineral salt basal (MSB) medium [26], supplemented with 0.1% sodium pyruvate as an extra carbon source. Kanamycin (100 μg/mL) was used to maintain the reporter plasmid.

2.3. Visualization and quantification of fluorescence response by the reporter bacterium

Ottawa sand and sandy loam samples aged with phenanthrene were prepared as described above. To identify the matrix effect of soil (i.e. growth inhibition), phenanthrene biodegradation by wild type *S. paucimobilis* EPA505 in the test soil was conducted, and the result was compared to the result of aqueous biodegradation test. Up to 90% of phenanthrene was degraded within 2 days, not only in the aqueous media with 500 mg/L of phenanthrene (as crystals), but also in the soil with 500 mg/kg-soil of phenanthrene. The viable bacterial cell numbers of the strain were also maintained at approximately 10^8 CFU/mL-medium (or gram-soil).