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Bioavailability of slow-desorbable naphthalene in a biological air sparging system

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Abstract. The bioavailability of sorbed organic contaminants is one of the most important factors used to determine their fate in the environment. This study was conducted to evaluate the bioavailability of slow-desorbable naphthalene in soils. An air sparging system was utilized to remove dissolved (or desorbed) naphthalene continuously and to limit the bacterial utilization of dissolved naphthalene. A biological air sparging system (air sparging system with bacteria) was developed to evaluate the bioavailability of the slow-desorption fraction in soils. Three different strains (*Pseudomonas putida* G7, *Pseudomonas* sp. CZ6 and *Burkholderia* sp. KM1) and two soils were used. Slow-desorbable naphthalene continuously decreased under air sparging; however, a greater decrease was observed in response to the biological air sparging system. Enhanced bioavailability was not observed in the Jangseong soil. Overall, the results of this study suggests that the removal rate of slow-desorbable contaminants may be enhanced by inoculation of degrading bacteria into an air sparging system during the remediation of contaminated soils. However, the enhanced bioavailability was found to depend more on the soil properties than the bacterial characteristics.

Keywords: bioavailability; slow-desorbable; biodegradation; soil slurry; aromatic contaminants

1. Introduction

Bioavailability is defined as the degree of the total concentration of a chemical that is available to microorganisms (Dudal *et al*. 2004). The bioavailability of organic contaminants is one of the most important factors used to determine their fate and toxicity in the environment (Oleszczuk 2009). It is generally accepted that sorbed contaminants desorb first, after which they become available for bacterial degradation. Accumulation of organic contaminants in soils would reduce their bioavilability to bacteria and decrease their biodegradation rate (Bosma *et al*. 1996, Harms and Bosma 1997). However, other studies have reported that bioavailability of sorbed contaminants was enhanced, with their biodegradation occurring more rapidly than desorption (Calvillo and Alexander 1996, Crocker *et al*. 1995, Guerin and Boyd 1992, 1993, Hwang and Cutright 2004, Ortega-Calvo and Saiz-

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Jimenez 1998, Park *et al*. 2003, Park *et al*. 2001, Poeton *et al*. 1999, Tang *et al*. 1998, Xia *et al*. 2010, Xia *et al*. 2006).

Xia *et al*. (2010) reported that desorption and biodegradation processes showed similar residual concentrations of sorbed phenanthrene in carbonaceous sorbents. However, the biodegradation rates were higher than the desorption rates during the rapid biodegradation stage, suggesting that bacteria could promote desorption or access and utilize the sorbed phenanthrene in carbonaceous sorbents, including black carbon and carbon nanotubes. In their study, batch experiments were utilized and a polystyrene resin Amberlite XAD-2 sorbent was applied to evaluate the abiotic desorption of phenanthrene from carbonaceous sediments. Hwang and Cutright (2004) suggested that microbial movement and adhesion to sorbent appeared to result in a great extent of sorbed PAH biodegradation when compared to the desorption and biodegradation of sorbed contaminants. Xia *et al*. (2006) observed an enhanced biodegradation rate of PAHs in the presence of sediment, suggesting that sorbed PAHs and the population of PAH-degrading bacteria increased as the amount of sediment in the solution increased, and that attached bacteria were exposed to a higher concentration of PAHs near the water-sediment interface, which increased the biodegradation rate. Poeton *et al*. (1999) also found an increased biodegradation rate of PAHs (phenanthrene and fluoranthene) with sediment content in solution, suggesting that bacterial interactions with sorbed PAH at the sediment particle surfaces increased the initial degradation rate in the presence of a sediment.

Most of these studies of bioavailability have focused on initial biodegradation of sorbed contaminants in the system. Therefore, there is little information available regarding the biodegradation of slow-desorbable contaminants in sorbents, which are equilibrated with very low liquid phase concentrations. Indeed, the liquid phase concentration does not properly indicate the bioavailability of sorbed contaminant, and contaminants are available to microbes at concentrations below the detection limit in the liquid phase.

This study was conducted to evaluate and understand the bioavailability of slow-desorbable contaminants in soils that are present at levels below the detection limit of the liquid phase concentration. To accomplish this, an air sparging system was utilized to remove dissolved (or desorbed) contaminants and maintain non-detectable liquid phase concentrations, while bacterial utilization of the dissolved contaminants was suppressed. Naphthalene was selected as the test contaminant because it has been often used as a model compound for PAHs, and this class of contaminants has a critical bioavailability issue at numerous petroleum contamination sites. Three naphthalene degrading bacteria and two soils were examined in this study.

2. Materials and methods

2.1 Bacteria and characteristics

The naphthalene degrading bacteria, CZ6 and KM1, were isolated from soil obtained from an industrial area highly contaminated with PAHs (Kim *et al*. 2007, Li *et al*. 2009). The naphthalene degrading bacterium, *Pseudomonas putida* G7, was obtained from C.S. Harwood at the University of Iowa.

The bacterial morphology and motility were observed using the easy flagellum staining method (Heimbrook *et al*. 1989). One drop of the cultivation solution was applied to a slide glass to examine the motility of the bacterium under a microscope (Olympus CX40). To observe the

morphology, staining solution that had been filtered using 0.45 μm membrane filters was added to the edge of the cover glass and the samples were then viewed and photographed using a Dino eyepiece digital camera (AM423X, Taiwan).

The bacteria were cultured in mineral salts medium (MSM) containing 3.4 g/L of KH_2PO_4, 3.55 g/L of Na_2HPO_4, 1.0 g/L of $(NH_4)_2SO_4$ and a 1% Hutner's mineral base (pH 6.8) (Harwood *et al*. 1994). Three strains were pre-cultured over night in Luria Bertani (LB) medium. For the main culture, naphthalene was added to the mineral salts medium as the sole carbon source at a concentration of 0.1% (w/v). Strains were cultured on a rotary shaking incubator at 150 rpm and 30°C. The bacterial culture was prepared to be at the exponential growth phase for seeding, after which it was washed three times with a PBS (Phosphate buffer saline) solution (Park *et al*. 2002). The cells were then diluted to an $O.D._{600nm}$ of 2.0 prior to inoculation into the air sparging system.

2.2 Chemicals and soil

Naphthalene was purchased from Sigma-Aldrich (98% purity). Stock solution was prepared in methanol. Heukyeumji and Jangseong soils were obtained from Jeju Island and Jangseong-gun, respectively, in South Korea. The organic carbon contents of Heukyeumji and Jangseong soils were 13.4 and 8.8%, respectively. The physicochemical properties of the two soils are presented in Table 1. The soil samples were air-dried and sieved to remove the soil fractions over 2 mm in diameter. The soils were sterilized by gamma irradiation (^{60}Co source) at a dose of 25 kGy prior to use.

2.3 Adhesion of the bacteria to soils

The assay used for the adhesion of bacteria to soil particles has been described previously (Huysman and Verstraete 1993, Mehmannavaz *et al*. 2001). The naphthalene degrading bacteria were cultured in liquid MSM medium with naphthalene as the carbon source. The bacterial culture was centrifuged to collect the cells. The pellet was washed three times in 150 mM NaCl solution and then adjusted to an optical density ($O.D._{600nm}$) of 0.7. Ten ml of free cells were added to 1.0 g of sterilized soil in a 50 ml test tube and vortexed for 60 s. After settling of the soil and attached bacteria for 10 min, 2 ml of the supernatant were transferred to a UV cell using a Pasteur pipette. The adhesion of the bacteria to the soils was calculated using the following equation: $\{[OD_{initial} - (OD_{final} - OD_{control})] \div ODinitial\} \times 100$. All experiments were performed in triplicate at room temperature.

2.4 Air sparging system

The experimental apparatus consisted of an air blower, a flow meter and ten air sparging vials (Fig. 1). Each flow meter was connected to an air sparging vial. The first air sparging vial contained

Table 1 Soil properties and the distribution coefficients of naphthalene

Soil	Kd	TOC[1)]	pH	Sand	Silt	Clay
Heukyeumji	10.3	13.4%	5.1	3.3%	76.8%	19.9%
Jangseong	10.4	8.8%	4.6	41.6%	56.1%	2.3%

[1)]TOC: total organic carbon

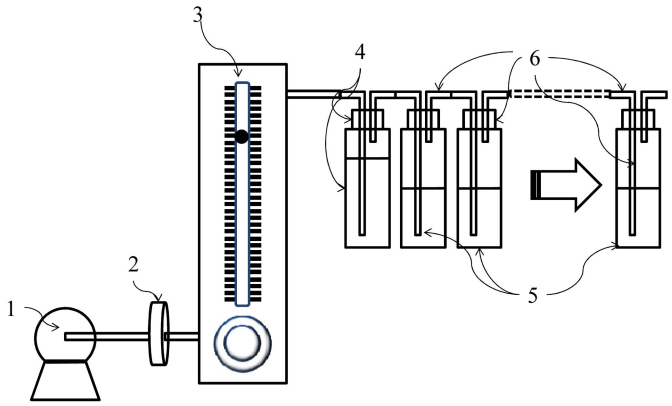


Fig. 1 Experimental apparatus for air sparging system (① Air blower; ② 0.2 μm Filter; ③ Flow meter; ④ Humidifier; ⑤ Soil slurry vials; ⑥ Air flow tubes)

15 ml of sterilized de-ionized water as a humidifier, which was connected to a flow meter and one of the air sparging vials. Vials were air sparged at a rate of 60 ml/min. Each 22 ml vial had an open screw cap with PTFE septa. Each slurry vial contained 0.2 g of sieved soil and 5 ml of MSM medium. Naphthalene stock solution was injected into the soil vials using a gas tight glass syringe. These samples were then left for two days in the dark at room temperature to achieve chemical sorption equilibrium by mixing at 4 rpm using a bench top roller (Wheaton).

2.5 Chemical analysis

To determine the concentrations of naphthalene in the soil slurries, each vial was centrifuged (1900 × g, 10 min) to separate the liquid and solid phases. The supernatant was then transferred to the 2 ml vials to determine the liquid phase concentration. Next, the sorbed contaminants in the soil were extracted by sonication for three minutes with 2 ml of methanol. The supernatant and extracted samples were then analyzed using HPLC with UV_{254nm} (YongLin M730D) and fluorescent detectors (Waters 2475) and the detection limit of naphthalene was 5 ppb in this study.

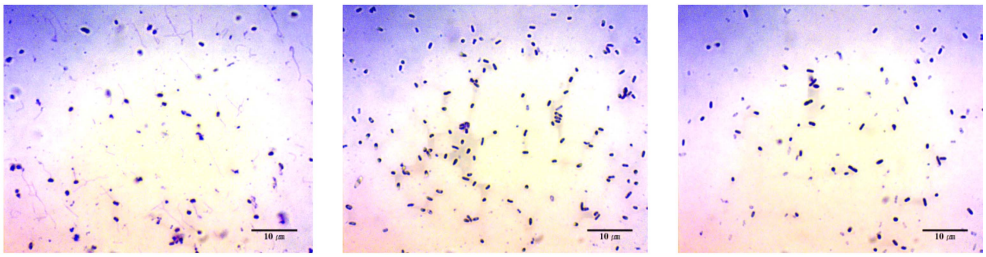
3. Results and discussion

3.1 Characteristics of bacteria

Fig. 2 shows stains of the three bacteria evaluated in this study, *Pseudomonas putida* G7, *Pseudomonas* sp. CZ6 and *Burkholderia* sp. KM1. Flagella were not observed for strains *Pseudomonas* sp. CZ6 and *Burkholderia* KM1, whereas *Pseudomonas putida* G7 had flagella. Motility was more strongly exhibited by *Pseudomonas putida* G7 than the others species, but was observed in all three strains.

3.2 Adhesion of bacteria to soil

Investigation of the adhesion of the three bacterial strains to soils revealed that strains G7 and



Pseudomonas putida G7 *Pseudomonas* sp. CZ6 *Burkholderia* sp. KM1

Fig. 2 Stained bacteria

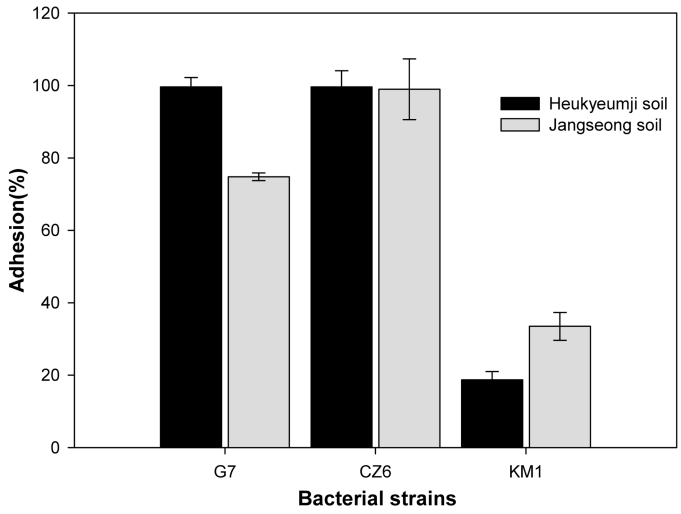


Fig. 3 Adhesion of bacteria to the soils. The error bars represent the standard deviation (n = 3).

CZ6 showed the highest adhesion to the two soils (Fig. 3). The adhesion values (>99%) indicated that almost all of these strains of bacteria had bound to the soils, indicating that strains G7 and CZ6 will be less mobile in the soil slurry system. Strain KM1 had the lowest adhesion to the two soils.

3.3 Effect of air flow rate on desorption

The controls in Figs. 4-5 show the results of desorption of sorbed naphthalene in air sparging systems. Initial rapid desorption occurred within 2 days, after which slow desorption occurred. After 2 days of desorption, no naphthalene was detected in the liquid phase, but the sorbed phase concentrations decreased with time. These findings indicate that naphthalene desorption from the sorbed phase to the liquid phase and stripping from the liquid phase to the gas phase occurred continuously, even though the liquid phase concentration was lower than the detectable concentration. In this study, sorbed naphthalene remaining after liquid phase concentrations in contact with the soils drop below the detection limit is defined as slow-desorbable naphthalene.

3.4 Biodegradation of slow-desorbable naphthalene in a biological air sparging system

To determine the bioavailability of slow-desorbable naphthalene in soils, a combination of three types of bacteria and two soils were utilized. As shown in Fig. 4(a), the decrease of the slow desorbable naphthalene fraction occurred more rapidly in the air sparging system with strain G7 (biological air sparging system) than without strain G7. After 17 days, the final concentration was lower in the system with strain G7 than without it. These results suggest that there is enhanced bioavailability of slow-desorbable naphthalene, which cannot be explained by biodegradation of the liquid phase contaminants. Several researchers have reported that the biodegradation rate was faster than the desorption rate of organic contaminants (Calvillo and Alexander 1996, Crocker *et al*. 1995, Guerin and Boyd 1992, 1993, Ortega-Calvo and Saiz-Jimenez 1998, Park *et al*. 2003, Park *et al*. 2001, Tang *et al*. 1998, Xia *et al*. 2010, Xia *et al*. 2006). These studies suggested that bacteria somehow utilize sorbed contaminants directly before desorption. However, all of these studies were performed in batch, without sparging. In the present study, an air sparging system was employed to remove dissolved (or desorbed) naphthalene continuously and to suppress the bacterial utilization of dissolved naphthalene. The more rapid removal of slow-desorbable naphthalene observed suggests that even though liquid phase contaminants are below the detection level, the removal rate of slow-desorbable (or strongly sorbed) contaminants can be enhanced by inoculation of degrading bacteria into an air sparing system during the remediation of contaminated soils. A similar result was observed on the seeding of strain CZ6 and KM1, as shown in Fig. 4(b) and 4(c), respectively. Following

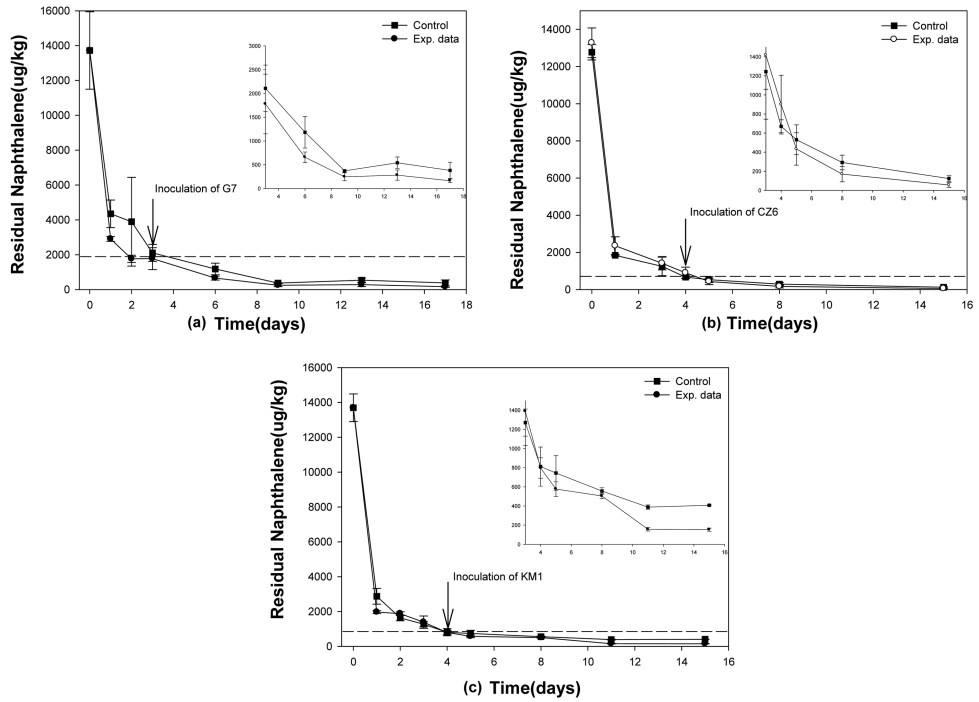


Fig. 4 Residual naphthalene in Heukyeumji soil in an air sparging system. Arrow indicates the point of strain inoculation. Liquid phase naphthalene was not detected below the dashed line. Flow rate: 60 ml/min; control: air sparging only. The error bars represent standard deviation (n = 3)

inoculation, the residual naphthalene in the soil decreased more rapidly than without inoculation. In both systems with and without inoculation, there were limitations in the removal of sorbed naphthalene within the experimental periods (15-17 days). In the present study, it was difficult to identify differences among the three bacteria with respect to the degradation of sorbed naphthalene in an air sparging system, although enhanced removal of slow-desorbable naphthalene was observed for all three biological systems with Heukyeumji soil. Guerin and Boyd (1992) reported different bioavailabilities of soil-sorbed naphthalene with two bacterial species (*Pseudomonas putida* ATCC 17484 and gram-negative strain NP-AlK), but this was not observed in the present study, even though there were different characteristics in the attachment, size and flagella of the bacteria.

In the Jangseong soil system, it was difficult to see the enhanced bioavailability of slow-desorbable naphthalene with all three strains (G7, CZ6 and KM1) (Fig. 5(a), (b) and (c)). The removal rate of the slow-desorbable naphthalene in the biological air sparging system was almost the same as the desorption rate in the air sparging system without bacteria. This result suggests that sorbed contaminants in Jangseong soil may not be directly available to bacteria, and that bacteria can only take up contaminants dissolved in the aqueous phase. Jangseong soil has a comparatively lower organic matter content, which may be related to the observation that the bioavailability of the slow-desorbable naphthalene was not enhanced. Ma *et al*. (2007) suggested that sorption of chemicals in soil with low organic matter occurs through pore filling and capillary condensation in minerals. Bacteria may not be able to access chemicals in the micropores of minerals.

It is difficult to explain the different phenomenon observed between the Heukyeumji and the

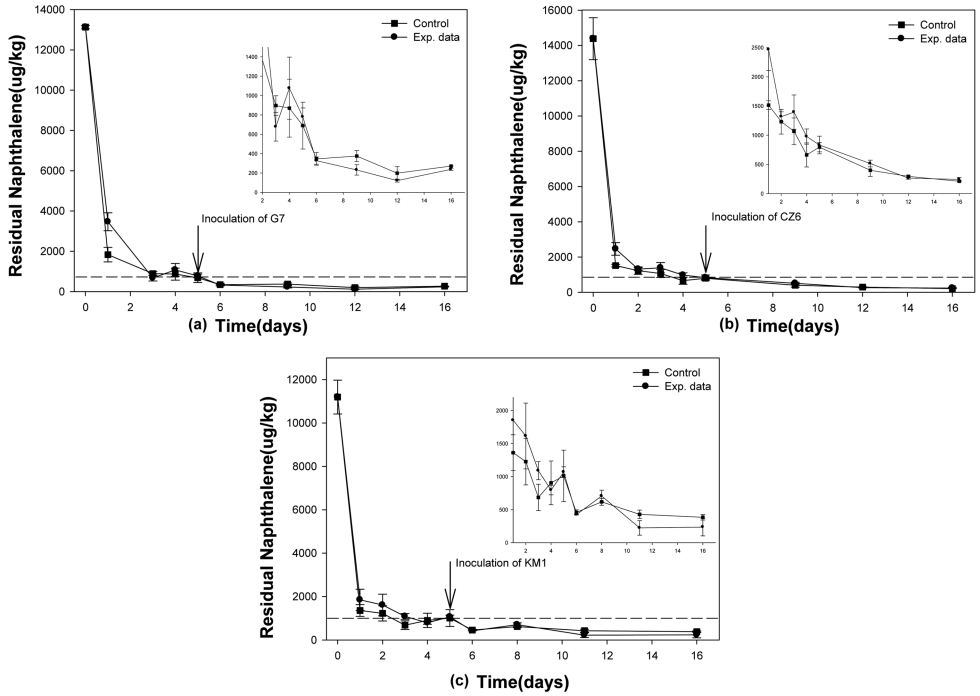


Fig. 5 Residual naphthalene in Jangseong soil in an air sparging system. Arrow indicates the point of strain inoculation. Liquid phase naphthalene was not detected below the dashed line; flow rate: 60 ml/min; control: air sparging only. The error bars represent standard deviation (n = 3)

Jangseong soil system. The naphthalene distribution coefficients were almost identical between the systems (Table 1), but the organic carbon contents were different. Several mechanisms on the enhanced bioavailability of sorbed contaminants have been speculated. First, bacterial adhesion may enhance the biodegradation of sorbed contaminants in soil organic matter by direct physical contact and chemical partitioning into the cell surface. Leglize *et al*. (2008) reported that bacterial adhesion onto activated carbon appeared to be related to the biodegradation of sorbed chemicals and that the bioavailability of sorbed contaminants was improved by higher bacterial adhesion capacity onto activated carbon. Park *et al*. (2002) reported enhanced bioavailability of sorbed naphthalene in soils with high organic matter. All bacteria used in this study had the ability to attach to soils, even though the adhesion differed among them (Fig. 3). These attachable bacteria may have accessed soil-sorbed naphthalene in Heukyeumji soil to enhance the bioavailability of slow-desorbable naphthalene. In this case, the contaminant does not need to be dissolved into a liquid phase to be biodegraded. Second, the mobility of chemotaxis bacteria may influence the degradation of sorbed contaminants in soils. The possible role of chemotaxis and mobility in bioavailability were examined in porous media, and Pedit *et al*. (2002) demonstrated that chemotaxis can increase the bioavailability in a porous matrix. Wild-type *Pseudomonas putida* G7 were found to increase the rate of naphthalene degradation when compared to the non chemotactic and non motile mutant strains (Law and Aitken 2003). In addition, wild-type bacteria with chemotaxis/mobility can access the non-desorbable fraction of naphthalene in activated carbon (Sajjad 2005). Generally, bacteria with flagella are more mobile than those without flagella. In this study, strain G7 had flagella and all of the strains had mobility (Fig. 2); therefore, the mobility of bacteria might have contributed to the observed enhanced bioavailability of slow-desorbable naphthalene in Heukyeumji soil (Fig. 4(a), (b) and (c)). Third, biosurfactants can extract contaminants from soils, reducing the distribution coefficient and increasing desorption and the bioavailability (Garcia-Junco *et al*. 2003, Herman *et al*. 1997, Oberbremer *et al*. 1990, Scheibenbogen *et al*. 1994). Fourth, the formation of a biofilm was found to enhance the bioavailability of phenanthrene, reducing the diffusion path length and improving the diffusion rate (Leglize *et al*. 2008). In our previous study (Li *et al*. 2009), the formation of an extracellular polymeric substance and biofilm by *Pseudomonas* sp. CZ6 was found to contribute to the increased biodegradation of sorbed naphthalene. In the present study, the bioavailability of sorbed naphthalene in soils was more affected by soil properties than bacterial characteristics. Non-desorbable naphthalene in soils with higher organic matter was more bioavailable than in soils with lower organic matter. These results suggest that the addition of degrading bacteria to contaminated soils with high organic matter will more effectively decrease the remediation time than simple air sparging. However, the mechanism of enhanced bioavailability of sorbed chemicals should be further researched.

4. Conclusions

This study was conducted to evaluate the bioavailability of slow-desorbable naphthalene in soils. To accomplish this, an air sparging system was utilized to remove dissolved (or desorbed) naphthalene continuously and to limit the bacterial utilization of dissolved naphthalene. Slow-desorbable naphthalene continuously decreased under the air sparging system; however, a greater decrease was observed in the biological air sparging system. All of the strains had the ability to enhance the removal rate of naphthalene from the slow-desorbable fraction in the Heukyeumji soil system, and they all biodegraded the contaminant more rapidly than it could dissolve to the aqueous phase.

However, enhanced bioavailability was not observed in Jangseong soil. These results suggest that the removal rate of slow-desorbable contaminants may be enhanced by inoculation of degrading bacteria into an air sparging system during the remediation of contaminated soils. However, the observation of enhanced bioavailability depends more on the soil properties than the bacterial characteristics.

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