PEROXIDASE-MEDIATED BINDING OF PHENOLS TO SOILS

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ABSTRACT

Sorption-desorption behavior of phenol and *o*-cresol was studied on two soils belonging to the Haynie series (very fine sandy loams) collected from field and forested sites. Experimental investigations focused on the effect of HRP addition on sorption and desorption of target chemicals when present in solution alone, and when present as a mixture. Addition of HRP resulted in a dramatic increase in sorption of the phenols. Desorption was little or negligible. No Fenton's oxidation of the target chemicals was observed. Hysteresis, represented by Hysteresis Indices, was significantly enhanced upon HRP addition. No competition was observed in dual-sorbate systems. Increases in Freundlich n and K_F values upon HRP addition were attributed to the production of hydrophobic polymers in the aqueous phase and new "organic matter" on the soil.

Key words: *sorption, desorption, enzyme, humification, hysteresis*

INTRODUCTION

Phenols have been classified as priority pollutants because of their multiple toxic health effects at very low concentrations (Clean Water Act, 1985). These chemicals constitute an important class of organic contaminants commonly associated with polluted soils and sediments. Phenols of anthropogenic origin may enter the environment as a result of uncontrolled discharges or accidental spills, or accumulate as intermediates during the incomplete biodegradation of aromatic compounds and pesticide mixtures (Guerin and Jones, 1988; Heitkamp and Cerniglia, 1988). Soil and sediments contaminated with such chemicals are a major cause of concern because of the high risks posed to ecosystem health.

Engineering remediation schemes to influence the fate and transport of organic contaminants in soils and sediments requires a thorough understanding of the governing physical, chemical, and biological processes in complex natural environments. Although sorption processes between organic chemicals and soil components have been well studied (Chiou et al., 1983; Pignatello, 1989; Weber and Huang, 1996), chemical reactions between these pollutants and soil/sediment matrices have not been investigated in great detail. There is increasing evidence that chemical interactions between organic pollutants and soil components, specifically reactions catalyzed by transition metal oxides or extracellular soil enzymes, can significantly affect the fate of contaminants in soils and sediments, and potentially alter the associated health risks from the chemicals (Wang et al., 1986; Voudrais and Reinhard, 1986; Nannipieri and Bollag, 1991; Bollag, 1992; Gianfreda and Bollag, 1994).

Soil organic matter (SOM) has been implicated as one of the fundamental factors controlling the fate of hydrophobic organic contaminants (HOCs) in soils and sediments (Chiou et al., 1983; Gschwend and Wu, 1985; Lee et al., 1990). Sorption capacities of soils for HOCs are highly

dependent on the content and chemical composition of SOM (Karickhoff et al., 1979; Garbarini and Lion, 1986; Gauthier et al., 1987; Gratwohl, 1990; Weber and Huang, 1996). In the case of ionogenic HOCs such as phenols, properties of the soil solution such as pH and ionic strength, as well as the acid dissociation constant (pK_a) of the chemical, can affect the pollutant's sorption behavior (Schellenberg et al., 1984; Westall et al., 1985; Lee et al., 1991).

Desorption processes have been observed to involve a relatively fast initial release of the sorbate followed by a prolonged and increasingly slower desorption, suggesting the possibility that a fraction of the solute may remain bound or sequestered in soil and sediment particles (DiToro and Horzempa, 1982; Steinberg et al., 1987; Pavlothasis and Mathavan, 1992; Xing et al., 1996). The desorption resistant fraction has been found to increase with increasing solute-sorbent contact times (Steinberg et al., 1987). The exact mechanisms responsible for sequestration and binding of solutes in soil matrices, however, remain unelucidated. In the case of phenols, the contaminants can be fixed or "trapped" within soil matrices as a result of enzymatic processes that imitate humus formation. There is increasing evidence that such chemical processes can significantly affect the fate of phenolic contaminants in soils and sediments, potentially altering the associated health risks from these xenobiotics (Wang et al., 1986; Voudrais and Reinhard, 1986; Nannipieri and Bollag, 1991; Bollag, 1992; Gianfreda and Bollag, 1994).

Soils contain a large background concentration of extracellular enzymes that catalyze degradation and biosynthesis reactions in the soil environment. These organic catalysts are often protected against natural degradation by their attachment to soil constituents. Several soil enzymes including peroxidases, laccases, and polyphenol oxidases, are capable of catalyzing chemical reactions that result in the polymerization of hydroxylated aromatic compounds. Sjoblad et al. (1976) were among the first to demonstrate the ability of a soil fungus derived phenoloxidase to polymerize hydroxylated aromatic chemicals. Suflita and Bollag (1980) later studied the polymerization of methoxyphenol and chloronaphthol by a soil-enzyme complex. Klibanov and coworkers utilized horseradish derived peroxidase to investigate the polymerization of aromatic amines and phenols in aqueous systems (Klibanov and Morris, 1981; Klibanov et al., 1983).

Enzyme-catalyzed oxidative coupling reactions can result in the formation of covalent linkages between phenols and humic substances. Bollag et al. (1980) observed the cross coupling of 2,4-dichlorophenol (DCP) to model humus constituents that included orcinol, syringic acid, vanillic acid, and vanillin. Bollag and Liu (1985) observed similar laccase-catalyzed copolymerization of syringic acid with other halogenated phenols including 4-chlorophenol, 2,6-DCP, 4-bromo-2-chlorophenol, 2,3,6- and 2,4,5-trichlorophenol, 2,3,5,6-tetrachlorophenol, and pentachlorophenol. Oxidative coupling catalyzed by three oxidoreductases—tyrosinase, peroxidase and laccase—was demonstrated for 2,4-DCP and a stream fulvic acid (Sarkar et al., 1988). This study also illustrated that enzyme- catalyzed incorporation of the target chemical into fulvic acid occurred over a wide range

of pH and temperature. Irreversible binding of aromatic compounds observed during sorption-desorption experiments has also been attributed to enzyme and transition metal oxide-catalyzed oxidative coupling of the contaminant to SOM (Bhandari et al., 1996; 1997; 1998; Burgos et al., 1996; 1999). Other researchers have investigated incorporation of pesticides such as atrazine into soils and have attributed a significant portion of the binding to oxidative polymerization reactions (Barriuso and Koskinen, 1996).

The work presented here represents selected early results obtained from a comprehensive investigation of enzyme-catalyzed binding of phenolic contaminants and their mixtures to natural geosorbents. Data obtained from natural system studies will be used to design engineered remediation systems that exploit the oxidative coupling properties of phenolic contaminants.

EXPERIMENTAL APPROACH

Soils

Two soils were collected near the city of Manhattan in Riley County, Kansas. These soils, collected from a forest site (SOM = 5.1%; pH = 7.1) and an agricultural site (SOM = 4.1%; pH = 7.3), belong to the Haynie series (fine sandy loam). The selection procedure was based on the U.S. Department of Agriculture's soil survey data and discussions with personnel at the Kansas Agricultural Experiment Station. Desired soil properties included near neutral pH, sandy/silty composition, and varying organic matter content and type. Once selected, the soils were collected aseptically and transported in coolers. Soils were sieved to pass through 1-mm and 500-mm sieves. Each soil was split into smaller representative fractions using the coning and quartering technique. Each soil fraction was sterilized using a multiple autoclaving procedure that included subjecting the soils to sequential autoclaving and incubation to neutralize all spore-forming bacteria. The soils were incubated twice for 48 hours and autoclaved three times for 50 minutes over a five-day period.

It was observed that when mixed with water, both soils released natural organic matter into solution. To avoid working with a three-phase system (soil, water, and dissolved organic material), we washed both soils several times to remove all leachable SOM. The soils (25 g) were washed with a synthetic groundwater (GW) solution (40 mL) consisting of a phosphate buffer and 500 mg/L of the biocide sodium azide (solution ionic strength = 18 mM). To remove all leachable SOM, the Haynie field soil was subjected to six washings while the Haynie forest soil was washed eleven times. The washed soils were dried at 70°C and homogenized with a mortar and pestle. The SOM content of the washed soils as determined by combustion at 550°C was 3.7% and 2.5% for the forest and field soils, respectively. All prepared soils were stored at sub-zero temperatures in glass bottles.

Target Chemicals

The target chemicals used in this study included uniformly labeled ^{14}C-phenol (specific activity: 14.3 mCi/mmol) and ^{14}C-*o*-cresol (specific activity: 4.8 mCi/mmol). These chemicals were purchased in their radiolabeled and nonlabeled forms from Sigma Chemicals and used without further purification. Known volumes of the radioactive target sorbate were added to a non-radioactive solution of the corresponding chemical. All solutions were prepared in the synthetic GW described previously. The non-labeled phenols were added from concentrated stock solutions prepared in methanol. To avoid cosolvency effects due to methanol, the volume of stock solution added was maintained at less than 0.1 % by volume. In studies involving phenol/cresol mixtures, parallel experiments were conducted with one target chemical being radiolabeled while the other chemical was nonlabeled. Radioactivity in aqueous samples was enumerated as disintegrations per minute (dpm) using a Beckman 6500 liquid scintillation counter (LSC) with quench and luminescence corrections. Horseradish peroxidase (Type II, RZ: 2.2) was purchased from Sigma Chemicals and used without further purification.

Adsorption-Desorption Experiments

Sorption-desorption experiments were conducted at three sorbate concentrations spanning two orders of magnitude. The initial aqueous concentrations were 5, 50, and 500 μM for both target sorbates. Preliminary investigations were conducted to determine the ideal solid/liquid ratio and solid/liquid contact time for adsorption experiments. From these studies, it was determined that each 16-mL glass centrifuge tube reactor would contain 5 grams of soil and enough solution to fill the tube with no headspace. A seven-day contact period was determined to be sufficient for equilibrium.

Constant solid dose adsorption experiments were conducted in glass centrifuge tubes operated as completely mixed batch reactors (CMBRs). Each CMBR contained 5 grams soil (dry weight basis) and approximately 14 mLs solution containing the radioactive and non-labeled sorbate. Each experimental setup included triplicate tubes consisting of the following: 1) HRP (soil + sorbate + enzyme + H_2O_2); 2) No HRP (soil + sorbate); 3) H_2O_2 control (soil + sorbate + H_2O_2); and 4) abiotic control (sorbate only). The "No HRP" experiments were conducted to quantify sorption-desorption properties of the soils when no enzyme was added. The "H_2O_2 control" tubes were included to assess changes in adsorption behavior due to the possibility of Fenton's oxidation upon peroxide addition to soil. The "abiotic control" tubes quantified contaminant losses due to volatilization or adsorption to reactor components.

To each "HRP" tube were added (i) five grams of the desired soil; (ii) approximately 12.5 mLs of 5, 50, or 500 μM solution of the target chemical (phenol or cresol, individually or as a mixture); (iii) 2 activity units (AU) of peroxidase per mL of solution, (one AU being the amount of enzyme that will form 1.0 mg of purpurogallin from pyrogallol in 20 seconds at pH 6.0 and 20°C.); and (iv)

sufficient H_2O_2 to achieve a solution concentration equal to the target chemical. The tubes were capped with teflon-lined phenolic caps and the contents mixed using a touch mixer. All tubes were then placed on their sides on a shaker table and allowed to equilibrate for seven days at 25 ± 1°C. During this contact period, the tubes were periodically removed from the shaker and their contents mixed using a touch mixer. At the end of 7 days, the tubes were removed from the shaker, their contents mixed on the touch mixture and then centrifuged at 250 *g* for 45 minutes to separate the solid and aqueous phases. A 250-µL aliquot was removed from each tube and transferred into a mini scintillation vial containing 5 mL scintillation fluid (ScintSafe Plus 50%, Fisher Scientific). The samples were allowed to sit overnight to minimize chemiluminescence and then analyzed for radioactivity using a LSC.

After the 250-µL aliquot was removed from the tubes at the end of the adsorption experiment, the soils were subjected to sequential "fill-and-draw" desorption as described elsewhere (Bhandari et al., 1997). The remaining supernatant was pipetted out and replaced with clean synthetic GW. The tubes were capped tightly, their contents mixed rapidly on the touch mixer, and placed on the shaker for overnight reequilibration (desorption). The tubes were removed from the shaker on the following day, their contents centrifuged, and the supernatant drawn for analysis on the LSC. Thereafter, fresh buffer was added, and the tubes reequilibrated and resampled daily until the radioactivity in the supernatant was reduced to below detection limit. The sorption-desorption experiments were repeated for all sorbent-sorbate-treatment combinations.

The adsorption data were fitted to the Freundlich model described as:

$$q_e = K_F C_e^n \quad or \quad \log q_e = n \log C_e + \log K_F \text{ (linearized form)}$$

where q_e and C_e represent the solid- and aqueous-phase concentrations of the target chemical, respectively. K_F is a measure of the sorption capacity of the solid at a specific aqueous concentration of the sorbate. The exponent n is a joint measure of the magnitude and heterogeneity of energy associated with the sorption process. Values of $n < 1$, represented by convex (Type I) isotherms, are indicative of adsorption by heterogeneous media where high energy sites are occupied first, followed by adsorption at sites with lower energies. Values of $n > 1$ represented by concave (Type III) isotherms, are indicative of adsorption behavior where previously sorbed molecules modify the surface such that further sorption of the sorbate is enhanced. Finally, linear (Type II) isotherms ($n = 1$) represent a phenomenon whereby the organic sorbate partitions into an amorphous SOM structure analogous to a separate organic phase. Both K_F and n also depend on sorbate properties, such as its octanol-water partition coefficient, K_{ow}.

Sorption-desorption behavior of organic contaminants in soils is often characterized by the occurrence of hysteresis. Possible reasons for this behavior include the presence of "ink-bottle" type pores that can trap the sorbate or, more likely, the occurrence of irreversible changes on the

sorbent surface resulting in a desorption process that is actually different from the adsorption process (Adamson, 1990). In this study, we measured hysteresis using the Hysteresis Index (HI) defined by Huang and Weber (1997) as:

$$HI = \frac{q_e^d - q_e^a}{q_e^a} | T, C_e$$

where q_e^a and q_e^d are solid-phase solute concentrations for the adsorption and desorption experiments, respectively; and T and C_e specify conditions of constant temperature and residual aqueous phase concentration. A HI of 1.0 is indicative of no hysteresis. HI values in the studies discussed here were calculated at C_e values of 10 μM.

RESULTS AND DISCUSSION

Single Sorbate Studies

Adsorption-desorption data for single sorbate systems are presented in Figure 1. A summary of results for the different soil-sorbate-treatment combinations studied is presented in Table 1. Figure 1(A) illustrates sorption-desorption behavior of phenol on Haynie field and forest soils. Adsorption isotherms were linear as indicated by the Freundlich *n* value of approximately 1.0 in both cases. This illustrates the partitioning nature of phenol in soils associated with diagenetically young SOM such as the ones used in this study. As a consequence of its marginally higher SOM content, the forest soil exhibited a slightly larger sorption capacity for phenol than the field soil. Desorption hysteresis was observed in both soils with the field soil manifesting a significantly greater hysteresis (HI = 10.2). The forest soil contained freshly decaying biomass and consequently a "younger" SOM as compared to the field soil. This may be the reason why less hysteresis was observed in the forest soil (HI = 3.8).

As seen from Figure 1(B), adsorption of cresol was near linear although the *n* values in this case were significantly smaller than for phenol. The sorption capacities, K_F, were higher in the case of cresol. This behavior is reflective of the greater hydrophobicity (larger K_{ow}) of *o*-cresol. Negligible desorption hysteresis was observed in this case. Isotherms resulting from the "H_2O_2 control" tubes (data not shown) were not significantly different from the "No HRP" tubes, indicating no Fenton's oxidation of the target chemicals by peroxide.

Figure 2 illustrates the effect of HRP addition to the sorption-desorption behavior of the target chemicals on field and forest soils. As is apparent from Figures 1 and 2 and Table 1, sorption capacities were increased several fold in all cases. This increase may be attributable to the simultaneous occurrence of two processes: (i) HRP-catalyzed production of phenolic polymers with reduced solubilites and greater hydrophobicities, and (ii) HRP catalyzed oxidative coupling or covalent bond formation between the parent phenols or phenolic polymers and SOM. In all cases, sorption linearity increased marginally or significantly upon HRP addition. This behavior is indicative

of sorption processes where previously sorbed molecules (phenolic polymers) may have modified the surface in such a way (for example, by increasing the surface hydrophobicity) that further sorption of the target chemical was increased. HRP-catalyzed "humification" reactions resulted in the production of hydrophobic polymers in the aqueous phase as well as an increase in the "organic matter content" of the soil, thereby enhancing sorption of phenols and their polymers.

The sorbed material was very resistant to desorption as illustrated in the increase in desorption hysteresis. The HI value for phenol sorption on forest and field soils increased from values of 3.8 and 10.2 to 17.6 and 19.7, respectively. In the case of cresol, the HI value increased from negligible to 11.9 and 15.6 for the forest and field soils, respectively. Another interesting observation was that in the absence of HRP, the sorption capacities for forest soil were greater than those for field soil. This was, however, reversed upon HRP addition. It appears as if the surface of the field soil was modified to a somewhat greater extent upon HRP addition, resulting in higher sorption in this case.

Dual Sorbate Studies

In studies involving phenol-cresol mixtures, parallel experiments were conducted with one target chemical being radiolabeled while the other chemical was nonlabeled. Adsorption-desorption isotherms for the phenol-cresol mixtures on the two soils are illustrated in Figures 3 (no HRP) and 4 (with HRP). Sorption-desorption parameters such as the Freundlich n and log $K_F$and the HI values are listed in Table 1. The data for phenol-field soil-no HRP, although presented here (Figure 3(A)), is suspected to be incorrect due to experimental errors and will not be discussed further. In all cases with no HRP, the sorption-desorption data were very similar to single-sorbate systems, indicating no competitive sorption. This corroborates our hypothesis that the organic matter is fairly "young" and amorphous and favors non-site dependent partitioning of the sorbates into the SOM "phase."

When HRP was added to the systems, once again a dramatic increase in sorption was observed. This increase was larger at higher aqueous concentrations than at lower concentrations reflected by higher Freundlich n values of 1.46 and 1.42 for phenol/cresol-Field soil, and 1.30 and 1.10 in for phenol/cresol-Forest soil in dual-sorbate systems (Table 1). The corresponding values in single-sorbate systems were 1.06, 1.15, 1.03, and 1.03 for phenol-Field, cresol-Field, phenol-Forest and cresol-Forest, respectively. Once again, desorption of sorbates was extremely reduced in the presence of HRP. Phenol was observed to desorb to a slightly greater extent than cresol, especially at the lowest concentration studied. HI values for dual sorbate systems in the presence of HRP were significantly higher than those for dual sorbate systems with no HRP or single sorbate systems with HRP (Table 1). It appears that sorption of both sorbates (in both soils) was enhanced when they were present as mixtures. This is an important observation as it points to the potential of peroxidase enzymes to effectively retard the mobility of phenolic mixtures in soils and groundwater.

CONCLUSIONS

The purpose of this paper was to present and discuss results from ongoing studies evaluating the effects of engineered humification processes on the fate of phenolic contaminants in soils and groundwater. Sorption-desorption of phenol and *o*-cresol was studied on two sandy loams with varying organic matter types and contents. Effect of the addition of peroxidase enzyme (and hydrogen peroxide) on the adsorption and desorption behavior of the phenols was evaluated. Finally, experiments were conducted to study sorption-desorption of the target chemicals in single-sorbate systems and as dual-sorbate mixtures. It was observed that sorption isotherms for all cases were linear or near-linear, indicative of the "young," amorphous nature of the SOM associated with the two soils. Although phenol exhibited some hysteresis in the absence of HRP, cresol showed little or no hysteresis for both soils. Addition of HRP resulted in dramatic increases in sorption for all soil-contaminant combinations. No Fenton's oxidation of the target chemicals was observed. Desorption was reduced to little or negligible. Hysteresis, as reflected by the HI values, was significantly enhanced upon HRP addition. No competition was observed in dual-sorbate systems. Freundlich n and K_F values increased with HRP addition possibly due to the production of hydrophobic polymers in the aqueous phase and simultaneous increases in the "organic matter content" as a result of enzyme-catalyzed oxidative coupling of phenols or polymers to SOM. Results of this study illustrate the capability of engineered humification processes, such as those catalyzed by peroxidase enzymes, to significantly alter the fate and transport of phenolic contaminants in soil, sediment, and groundwater systems.

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Table 1. Freundlich isotherm parameters (n and log K_F) and Hysteresis Index (HI) values for the different soil-contaminant-treatment combinations evaluated. HRP = horseradish peroxidase enzyme. HI values were determined using q_e^a and q_e^d values at C_e = 10μM.

Soil	Target-Chemical	HRP	n	log K_F	R^2	HI
Haynie-Field	Phenol	No Yes	1.00 1.06	-0.75 0.13	0.99 1.00	10.2 19.7
	Cresol	No Yes	0.88 1.15	-0.30 0.33	0.99 0.99	++ 15.6
Haynie-Forest	Phenol	No Yes	1.02 1.03	-0.65 0.00	0.99 1.00	3.8 17.6
	Cresol	No Yes	0.92 1.03	-0.24 0.21	0.99 0.99	++ 11.9
Haynie-Field	Phenol in Mix	No Yes	0.55* 1.46	-0.24* -0.63	0.95* 0.95	++ 58.0
	Cresol in Mix	No Yes	0.79 1.42	-0.14 0.28	0.98 0.97	++ 14.9
Haynie-Forest	Phenol in Mix	No Yes	1.02 1.30	-0.59 -0.58	0.99 0.97	6.4 29.1
	Cresol in Mix	No Yes	0.93 1.10	-0.25 0.22	0.98 0.99	3.7 20.1

*Data suspected to be incorrect due to experimental errors (field soil, phenol in mixture, no HRP).
++HI values not calculated due to little or no hysteresis.

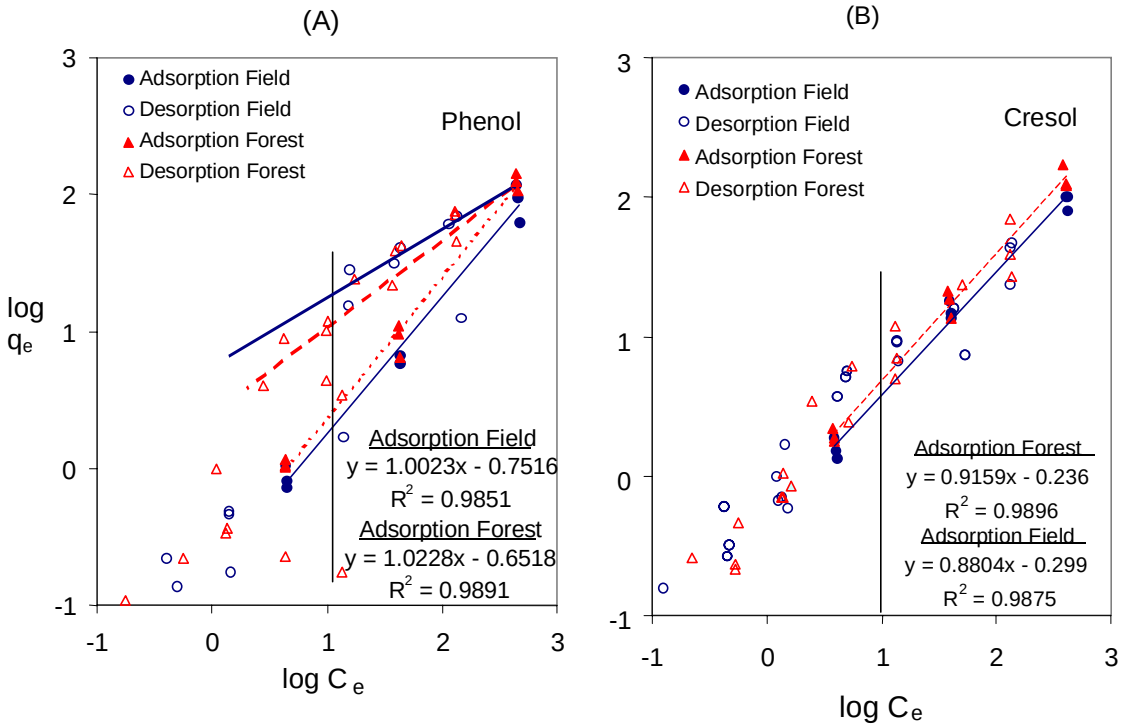

Figure 1. Adsorption-desorption isotherms for individual target chemicals (A) phenol and (B) o-cresol on Haynie field and Haynie forest soils.

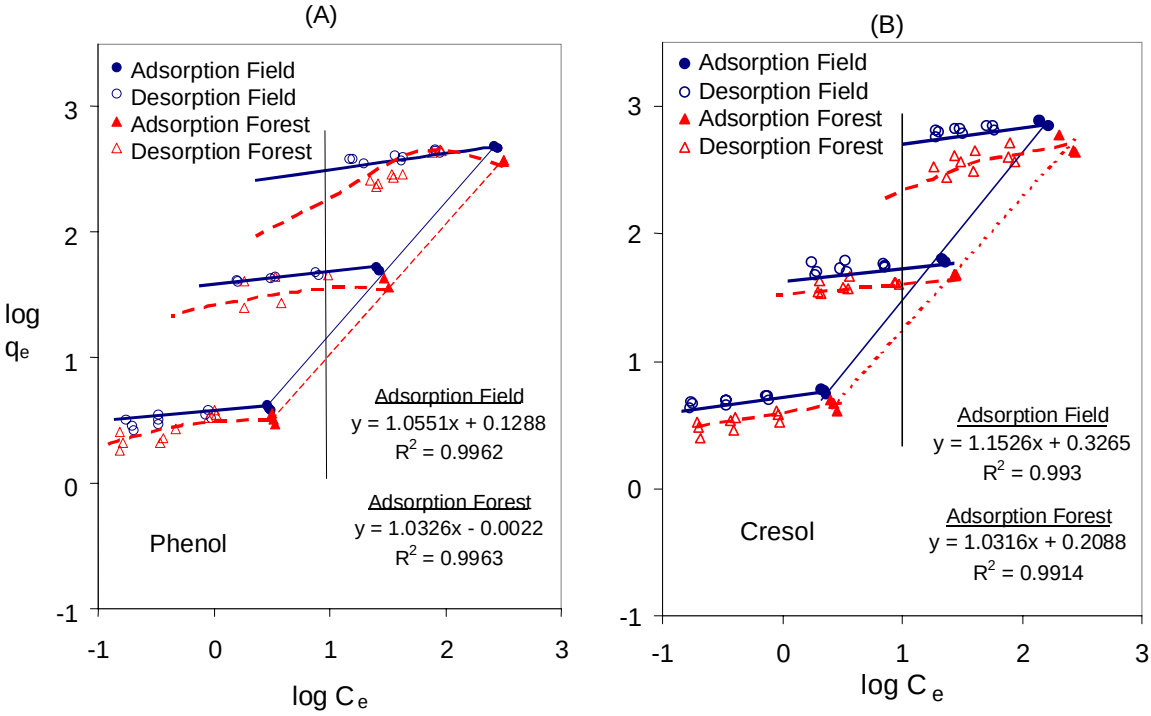

Figure 2. Effect of horseradish peroxidase addition on adsorption-desorption isotherms for individual target chemicals (A) phenol and (B) *o*-cresol on Haynie field and Haynie forest soils.

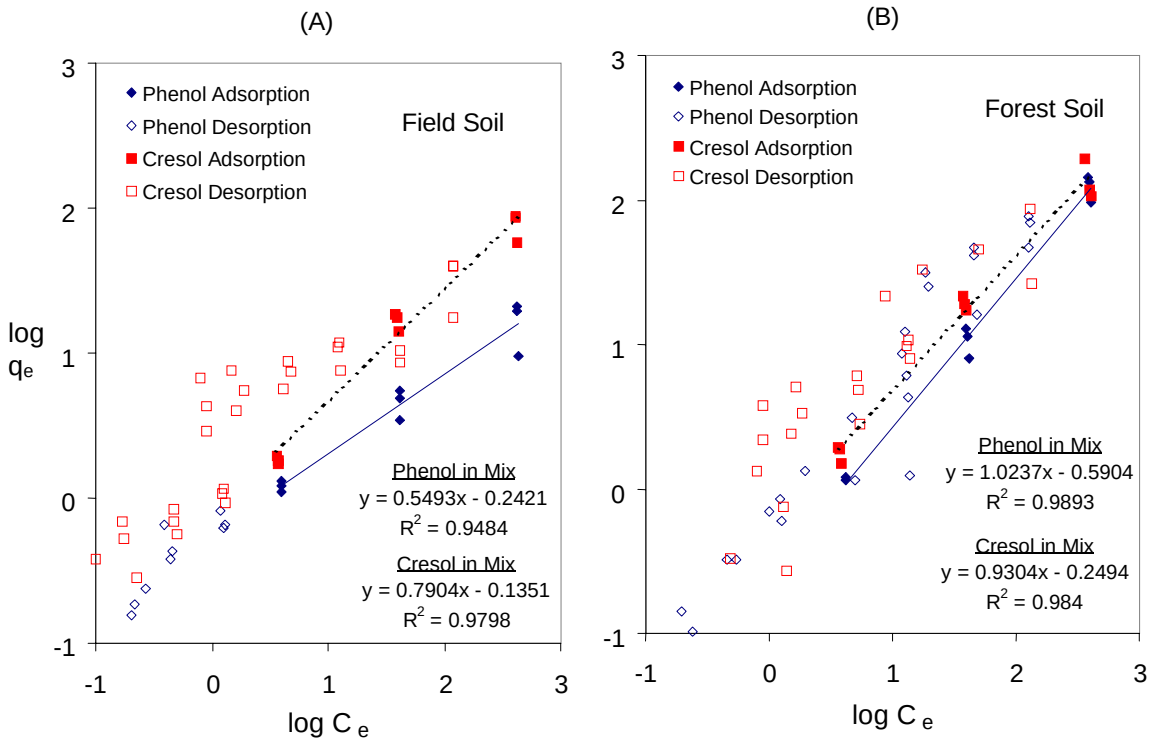

Figure 3. Adsorption-desorption isotherms for phenol-cresol mixtures on (A) Haynie field and (B) Haynie forest soils.

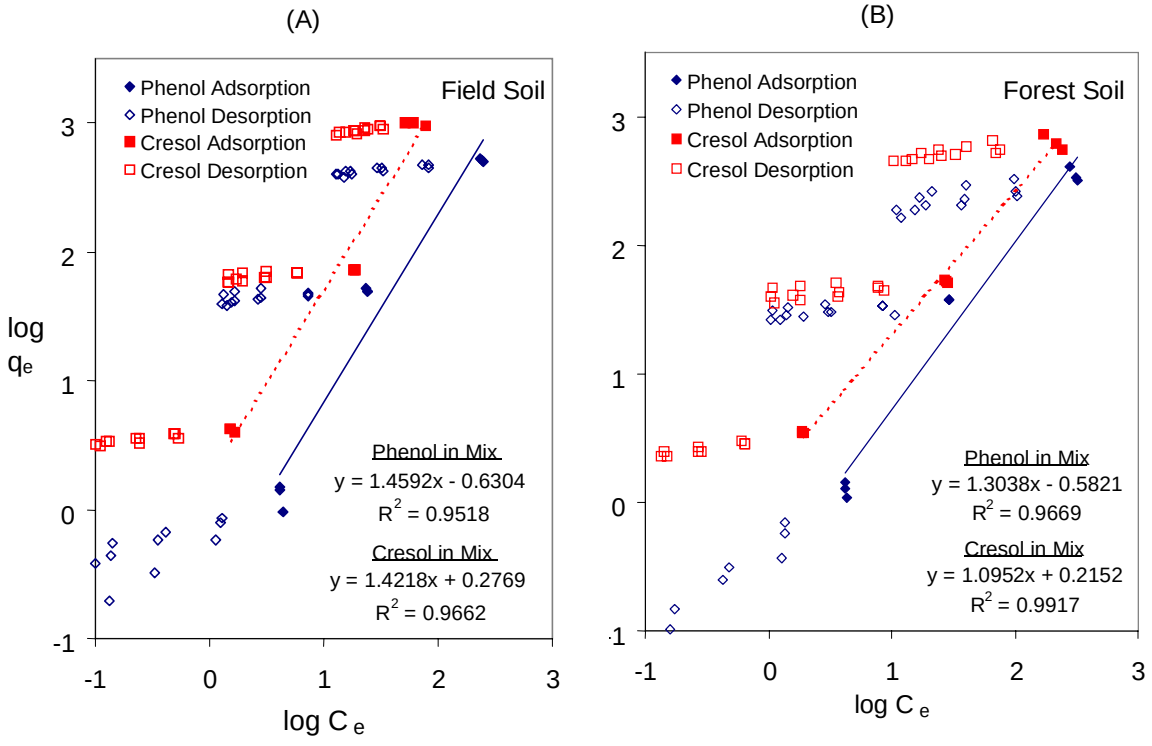

Figure 4. Effect of horseradish peroxidase addition on adsorption-desorption isotherms for phenol-cresol mixtures on (A) Haynie field and (B) Haynie forest soils.