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Influence of a soil enzyme on iron-cyanide complex speciation and mineral adsorption

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Abstract

Cyanide is commonly found as ferrocyanide $[Fe^{II}(CN)_6]^{-4}$ and in the more mobile form, ferricyanide $[Fe^{III}(CN)_6]^{-3}$ in contaminated soils and sediments. Although soil minerals may influence ferrocyanide speciation, and thus mobility, the possible influence of soil enzymes has not been examined. In a series of experiments conducted under a range of soil-like conditions, laccase, a phenoloxidase enzyme derived from the fungi *Trametes versicolor*, was found to exert a large influence on iron-cyanide speciation and mobility. In the presence of laccase, up to 93% of ferrocyanide (36–362 ppm) was oxidized to ferricyanide within 4 h. No significant effect of pH (3.6 and 6.2) or initial ferrocyanide concentration on the extent or rate of oxidation was found and ferrocyanide oxidation did not occur in the absence of laccase. Relative to iron-cyanide–mineral systems without laccase, ferrocyanide adsorption to aluminum hydroxide and montmorillonite decreased in the presence of laccase and was similar to or somewhat greater than that of ferricyanide without laccase. Laccase-catalyzed conversion of ferrocyanide to ferricyanide was extensive though up to 33% of the enzyme was mineral-bound. These results demonstrate that soil enzymes can play a major role in ferrocyanide speciation and mobility. Biotic soil components must be considered as highly effective oxidation catalysts that may alter the mobility of metals and metal complexes in soil. Immobilized enzymes should also be considered for use in soil metal remediation efforts.

Keywords: Laccase; Cyanide; Ferrocyanide; Metal adsorption; Aluminum hydroxide

1. Introduction

Cyanide is a common soil contaminant and is often found associated with present or former manufactured gas, electroplating plants, and precious metal mining sites and is known to be toxic to most organisms. At such sites, cyanide is commonly found in soils complexed with iron as ferrocyanide $[Fe^{II}(CN)_6]^{-4}$ and ferricyanide $[Fe^{III}(CN)_6]^{-3}$ (Mansfeldt et al., 2004). Although both forms are much less toxic than free cyanide (CN^-), they are potentially hazardous because CN^- can be released from these compounds by photolysis (Meeussen et al., 1992). Evidence suggests that ferricyanide is more mobile in soils due to its generally greater solubility under most soil conditions and is a more kinetically labile complex (Cheng et al., 2000; Rennert and Mansfeldt, 2002a,b, 2005). Ferricyanide, therefore, has a greater potential to contaminate groundwaters and release toxic CN^- than ferrocyanide (Meeussen et al., 1992; Rennert and Mansfeldt, 2002a,b). Thus, it is important to understand the processes that control the speciation of iron-cyanide complexes in soil.

Oxidation/reduction reactions in soils may be controlled by abiotic (minerals) or biogenic (organic compounds

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including enzymes) soil components, either as terminal electron acceptors and donors or as mediators of electron transfer (catalysts). For example, previous research has shown that a soil mineral, manganese dioxide, can oxidize ferrocyanide to ferricyanide (Rennert et al., 2005b) acting as a terminal electron acceptor. However, there has been no research identifying biogenic agents of ferrocyanide oxidation, or more specifically, the possible role of enzymes in mediating electron transfer to and from soil metals.

One of the main groups of soil enzymes, the oxidoreductases, including such extracellular enzyme as laccase, tyrosinase, and peroxidase, are known to participate in a variety of transformation reactions occurring in soil, including transformation of anthropogenic chemicals. As such, there has been a great deal of research investigating the theory and practical aspects of their use in contaminated soil remediation. Although some extracellular enzymes are known to mediate the oxidation of many organic compounds via the reduction of molecular oxygen (Dick and Tabatabai, 1993; Dec and Bollag, 2000; Xu et al., 2000; Ahn et al., 2002, 2006), there is relatively less information on their possible role in oxidation of inorganic compounds. In one set of studies, the kinetics of horseradish peroxidase was studied via ferrocyanide oxidation (Cotton and Dunford, 1973; Cotton et al., 1973). In addition, several metal ions have been shown to stimulate the activity of certain soil enzymes (Singh and Tabatabai, 1978; Dick and Tabatabai, 1983). Therefore, it may be that many enzymes have the ability to alter the speciation of inorganic soil components.

Laccase is an oxidoreductase that possesses high oxidation capacity due to its ability to transfer electrons between copper in the laccase molecule and target chemicals (Gianfreda and Bollag, 2002). With a redox potential of 775–780 mV (Xu et al., 1996; Gianfreda and Bollag, 2002), *Trametes* sp. laccase has the potential to oxidize ferrocyanide in soils (ferrocyanide $\rightarrow$ ferricyanide $+ e^-$, 356 mV: Rennert and Mansfeldt, 2005).

The mobility of metal complexes in soils is controlled by their affinity for soil component surfaces. Iron-cyanide complexes are known to sorb to a variety of surfaces including clay, iron and aluminum oxyhydroxides, organic matter and even quartz sand, though sparingly (Rennert and Mansfeldt, 2002b; Bushey and Dzombak, 2004). However, unlike abiotic catalysts (such as manganese dioxides), enzymes too, often have high affinity for mineral surfaces and may, therefore, compete with iron-cyanide complexes for mineral surface adsorption sites. Proteins, such as enzymes, have been shown to adsorb onto soil constituents (e.g. Claus and Filip, 1988; Naidja et al., 1997; Zimmerman et al., 2004a). The identified mechanisms of enzyme adsorption onto clay minerals include cation exchange, electrostatic attraction, and hydrophobic binding (Nannipieri et al., 2002). Another chemical phenomena to consider, is the possible inactivation of enzyme oxidation–mediation ability through enzyme–mineral interaction. While some enzymes are inactivated by mineral adsorption, others retain or even show enhanced activity when in their mineral-adsorbed state (Gianfreda et al., 1992; Naidja et al., 2000; Gianfreda and Bollag, 2002; Nannipieri et al., 2002).

Enzyme–mineral–iron-cyanide interaction experiments were conducted to evaluate the relationship between abiotic and biotic mechanisms related to cyanide mobility in soils. Specifically, oxidative transformation and adsorption experiments were carried out to examine the influence of a fungal enzyme, laccase from *Trametes villosa*, on ferrocyanide oxidation and adsorption onto aluminum hydroxide and montmorillonite. Previous work has implicated oxalate-extractable Al and clay content as among the most important soil property influencing iron-cyanide sorption (Rennert and Mansfeldt, 2002b). However, while the iron-cyanide sorptive properties of crystalline Al oxyhydroxides have been studied (γ-Al_2O_3: Cheng and Huang, 1996; Bushey and Dzombak, 2004), no previous work has examined that of poorly ordered Al hydroxides, which is likely to be the most significant soil Al oxide phase in terms of reactive surface area. Although clays make up a quantitatively important portion of mineral surface area in soils, their influence on iron-cyanide mobility has not previously been examined in this context. Montmorillonite was chosen for study here as it is one of the most common soil clay minerals. The goal of this study is an improved understanding of the synergistic effects that occur in a model ternary system containing the enzyme laccase, minerals and iron-cyanide to access the likely mobility of iron-cyanide in contaminated soils.

2. Materials and methods

2.1. Materials

Ferrocyanide as $K_4Fe(CN)_6$, ferricyanide as $K_3Fe(CN)_6$ (both 99.0% purity), and laccase (EC 1.10.3.2) from *Trametes versicolor* were obtained from Sigma–Aldrich Co. Montmorillonite was obtained from the Clay Minerals Society (Chantilly, VA). The <2 μm fraction was prepared by sieving and washing with 0.5 M KCl solutions at least four times. Excess KCl was removed by dialysis against distilled water until no further chloride ion was extracted. The montmorillonite suspension was freeze-dried and stored in a closed container prior to use.

Poorly crystalline aluminum hydroxide was synthesized by gradual neutralization of a 0.5 M $AlCl_3$ solution to pH 7.0 using 0.5 M NaOH (following Huang et al., 1977). The suspension was aged for 48 h at room temperature and centrifuged at 10,300g for 20 min. After removal of the supernatant, the precipitate was washed with deionized water to completely remove Cl^- and freeze-dried.

The specific surface area of aluminum hydroxide and montmorillonite were 297.5 and 41.4 $m^2\ g^{-1}$, respectively, as determined using N_2 sorptometry on a Micromeritics ASAP 2021 sorptometer (Norcross, GA) from the linear segment of the multi-point N_2 adsorption isotherms. The poorly crystalline nature of the Al hydroxide was confirmed by X-ray diffraction analysis.

2.2. Oxidation experiments

Laccase (0–311 μg ml^{-1}) was added to ferrocyanide (36–362 μg ml^{-1}, in 20 ml 0.1% NaCl) under various pH conditions (pH 3.6–6.2). Given the definition of one unit of activity as the capacity to oxidize one mol catechol per min at pH 4.5 and 25 °C, the range of laccase concentration used in this study is equivalent to 0–7.37 units of activity. All experiments were conducted in triplicate.

After 48 h in a rotating mixer (or less in the case of time course experiments), samples were removed and filtered through 0.25 μm mesh nylon filters prior to ferrocyanide analysis using the 'ferric chloride indicator' method of McGivney and Shelton (1984) as follows. After acidification with concentrated HCl (to pH < 2), 100 μl of 0.111 M $FeCl_3$ solution was added to 3.5 ml of the filtrate. After 12.5 min, samples were colorimetrically analyzed by a UV spectrometer at 700 nm. This method detects only ferrocyanide along with its conjugate acids. Response was calibrated at each pH in the presence of ferricyanide and checked with occasional additional measurements of total cyanide (by distillation described below).

2.3. Adsorption experiments

Adsorption of ferrocyanide and ferricyanide on Al hydroxide and montmorillonite was conducted at pH 3.7 ± 0.1 and 6.2 ± 0.1 in the presence and absence of laccase (204 μg ml^{-1}). The pH of all samples was adjusted by adding dilute HNO_3 or NaOH. Various amounts of ferricyanide or ferrocyanide (from 10 to 50 μg ml^{-1}) in 20 ml 0.1% NaCl solution were combined with 0.2 g Al hydroxide or montmorillonite in 50 ml centrifuge tubes covered with aluminum foil to prevent photodegradation. The mixtures were placed in an end-over-end rotator for 48 h when adsorption equilibrium was determined to have been reached. Each treatment was conducted in duplicate tubes. After centrifugation (5000 rpm for 10 min) and filtration through a 0.25 μm nylon membrane, total cyanide was extracted from the supernatants and concentration of total iron-cyanide was determined using ion chromatography (described below). Experiments accessing adsorption of laccase (without cyanide compounds) on Al hydroxide or montmorillonite were conducted similarly (using 204 μg ml^{-1} laccase in 20 ml 0.1% NaCl solution with 0.2 g mineral). Amount adsorbed was calculated as the difference between the sorptive concentration in solutions in tubes containing the mineral and control tubes containing the sorptive and no mineral. Sorption data was modeled using the Freundlich equation.

2.4. Total cyanide analysis

For total cyanide analysis, supernatants were digested and distilled using standard distillation techniques for total cyanide (APHA, 1998). All iron-cyanide complexes in the supernatants were converted to hydrogen cyanide by the addition of 50 ml 50% HCl solution and purged with air. The resultant HCN gas was collected using a NaOH scrubbing solution and was quantified by ion chromatography on a Dionex 600 Ion Chromatograph with an ED40 electrochemical detector via direct injection. This method has a typical detection limit of 10 μg l^{-1} and a working range of 30–1000 μg l^{-1}.

2.5. Laccase analysis

A protein assay, Bio-Rad reagent from Bio-Rad Lab. (Richmond, CA), was used to determine laccase concentration in supernatants following adsorption experiments. A calibration curve was prepared using bovine serum albumin as a standard. Colorimetric analyses of the Bio-Rad-protein complex formed were carried out using a Model UV-1601 spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) operated at 595 nm.

3. Results and discussion

3.1. Oxidation experiments

The temporal stability of dissolved ferrocyanide in the presence and absence of laccase is shown in Fig. 1a. In the absence of laccase, no loss, i.e. conversion, of ferrocyanide occurred showing that O_2 in the study system has negligible effect on ferrocyanide oxidation. For all three ferrocyanide concentrations tested, more than 80% of ferrocyanide was transformed within 4 h in the presence of laccase, and laccase was capable of nearly completely removing ferrocyanide within 24 h. No significant transformation of ferrocyanide occurred during this time in control experiments without laccase. Within the linear portion of the reaction (first 20 min), the specific activity of ferrocyanide conversion by laccase was 23.5 μmol mg^{-1} min^{-1}.

Only a small pH effect on extent of ferrocyanide conversion was observed in the range of pH 3.1–8.4. However, the maximal ferrocyanide conversion in the pH 6.5–8 range (Fig. 1b) is notably higher than the optimal pH for phenol oxidation activity commonly reported of pH 4–5 (Ahn et al., 2007).

Laccase quantity was observed to have a small but significant effect on ferrocyanide conversion (Fig. 1c). While more than 80% of 1.6 mg ferrocyanide (78 μg ml^{-1}) was converted by 1 mg (50 μg ml^{-1}) laccase within 48 h, 3 mg laccase was required to convert 7.2 mg (362 μg ml^{-1}) ferrocyanide to the same degree. In the former case, 243 mol ferrocyanide was converted per mol laccase, and 374 mol ferrocyanide was converted per mol laccase in the latter. These data indicate that laccase is acting here as a catalyst of ferrocyanide oxidation, transferring electrons to and from its copper site (likely via dissolved O_2), and not simply acting as a one-time oxidation partner. Given that 362 μg ml^{-1} is a concentration of ferrocyanide is likely to be found in contaminated soils (Kjeldsen, 1999), a small

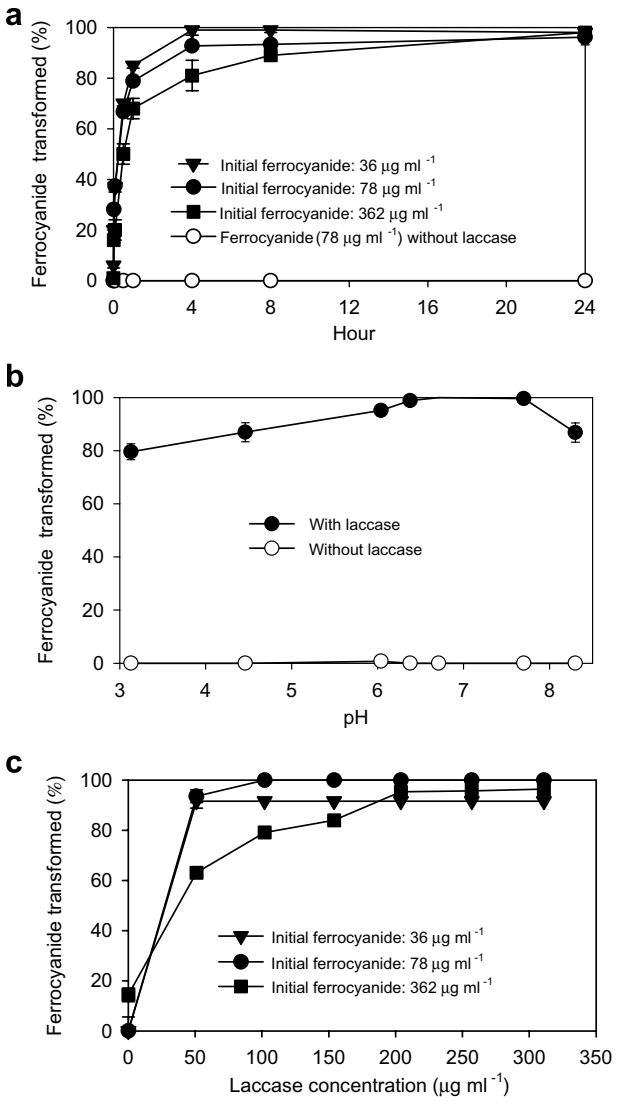

Fig. 1. Transformation of ferrocyanide in the presence of *T. versicolor* laccase as a function of (a) reaction time and initial ferrocyanide concentration (204 μg ml^{-1} laccase, pH 6.2), (b) pH (204 μg ml^{-1} laccase, 78 μg l^{-1} initial ferrocyanide concentration, 48 h), and (c) initial concentration of laccase and ferrocyanide (pH 6.2, 48 h).

amount of laccase can, apparently, have a great effect on iron-cyanide speciation in most soil systems.

While, ferricyanide was not directly analyzed, it is the most likely the major product of the laccase-mediated oxidation of ferrocyanide for the following reasons. First, total cyanide remained constant so it is unlikely iron-cyanide degradation took place. Second, laccase, with a redox potential of 775 mV is thermodynamically able to oxidize ferrocyanide to ferricyanide ($E_H = 356$ mV: Rennert et al., 2005a). Third, although free cyanide CN^- is the thermodynamically predicted predominate species at the experimental conditions, its formation is kinetically limited (Meeussen et al., 1994) as it is in soils and groundwater. Samples were not exposed to light, which is considered necessary for conversion of iron-complexed cyanide to free cyanide at the timescale of these experiments (Meeussen et al., 1994). Lastly, under aerobic conditions ($E_H >$ 400 mV), ferrocyanide has been shown to almost completely oxidize to ferricyanide after 250 h (Rennert and Mansfeldt, 2005). The results show the thermodynamically favorable and kinetically likely oxidation of ferrocyanide to ferricyanide to be catalyzed by the phenoloxidase laccase enzyme. In soil-like solutions in the presence of laccase, the oxidation occurs readily and to completion. However, the importance of this process in the soil environment will depend upon the interaction of laccase and iron-cyanide species with soil minerals.

3.2. Iron-cyanide adsorption experiments

To evaluate the possible effects of minerals on iron-cyanide mobility in soil systems, adsorption experiments were performed on poorly ordered Al hydroxide and montmorillonite in the presence and absence of laccase. In the absence of laccase, Al hydroxide adsorbed both ferrocyanide and ferricyanide at pH 3.7 and 6.2, while montmorillonite only adsorbed significant ferrocyanide, and only at pH 3.7 (Fig. 2). It should be noted that no evidence was found for ferrocyanide oxidation by contact with Al hydroxide or montmorillonite alone. Maximum observed sorbed concentrations were 2.9–4.9 mg g^{-1} iron-cyanide on Al hydroxide, and 0.3–2.7 mg g^{-1} iron-cyanide on

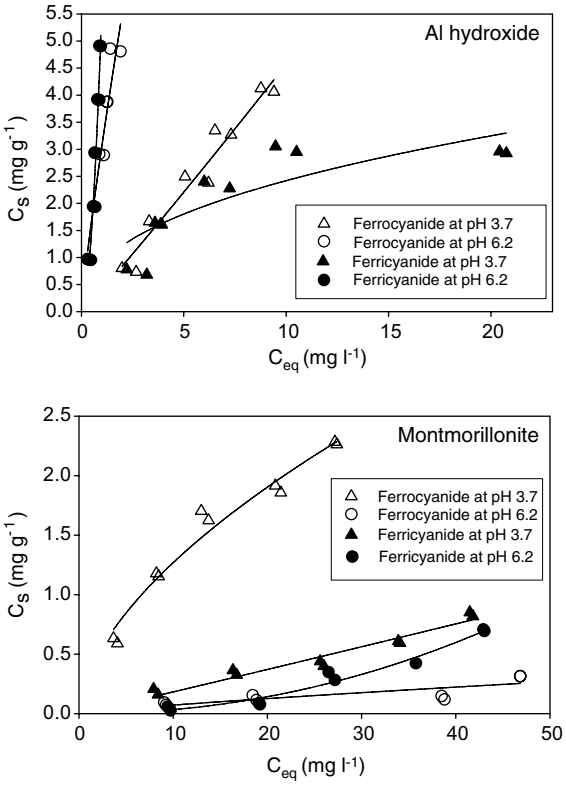

Fig. 2. Adsorption isotherms of ferrocyanide and ferricyanide on Al hydroxide and montmorillonite, at pH 3.7 and 6.2 in the absence of laccase. Freundlich equation isotherm lines are plotted.

montmorillonite. The iron-cyanide sorption capacity of this Al hydroxide is in the mid-range of that previously reported for Al or Fe oxyhydroxides (0.005–40 mg g^{-1}: e.g. Bushey and Dzombak, 2004), likely due to its high surface area and reactive site density relative to that of more crystalline oxyhydroxides (Hsu, 1989). No quantitative data on iron-cyanide/clay adsorption has been previously published with which to compare our results.

Adsorption isotherms were generally well-described by the Freundlich model; $C_s = K_f C_{eq}^{1/n}$, where C_s is the concentration of sorptive associated with the sorbent, C_{eq} is the sorptive concentration in solution, K_f is the Freundlich adsorption constant; and n is the Freundlich exponent. The r^2 values ranged 0.65–0.98 in the absence of laccase (Table 1). However, three of the isotherms in Fig. 2 display a distinct sorption plateau and are, therefore, better fit using a Langmuir isotherm. Using the Langmuir equation, maximum sorption capacities of 4.2 mg g^{-1} for ferricyanide on Al hydroxide at pH 3.7, and 3.7 and 0.1 mg g^{-1} for ferrocyanide at pH 3.7 and pH 6.2 on montmorillonite, respectively, were calculated.

Significantly greater adsorption of both ferro- and ferrcyanide were observed by Al hydroxide (K_f: 0.337–5.74, Table 1) than by monrtmorillonite (K_f: 0.001–0.271, Table 1). These results implicate electrostatic attraction as the most important adsorption mechanism for both the ferrocyanide and ferricyanide anionic complex. The point of zero net charge (pznc), the pH at which the number of negative and positive surface charge sites are balanced, is 8.0–9.2 for short-range ordered Al hydroxide (Hsu, 1989). Therefore, the net surface charge of Al hydroxide will be predominantly positive ($AlOH_2^+ > AlO^-$) at both experimental conditions. The surface charge of montmorillonite, however, with a pznc of 2–3, will have fewer positively charged sites and only at lower pH.

Previous research has reported a trend of increasing adsorption with decreasing pH for both ferrocyanide and ferricyanide adsorption onto soils and Al oxides (Cheng and Huang, 1996), Fe oxides (Rennert and Mansfeldt, 2002a), and Mn oxides (Rennert et al., 2005b). These findings support outer-sphere complexation as the predominant binding mechanism proposed for iron-cyanide adsorption onto minerals (Cheng et al., 2000).

Greater ferro- and ferricyanide sorption by montmorillonite was observed at pH 3.7 than pH 6.2. This trend can be seen both in the Freundlich isotherm distribution coefficients (Table 1), i.e. greater affinity of ferro- and ferricyanide for montormillonite, as well as the greater apparent adsorption capacity of ferrocyanide at each pH (plateau adsorbed concentration modeled by Langmuir isotherms). However, contrary to expectations, both ferrocyanide and ferricyanide displayed greater adsorption to Al hydroxide at pH 6.2 than pH 3.7. The dissociation constants of ferricyanide are all below 1, but the pK_3 and pK_4 of ferrocyanide are 2.2 and 4.2, respectively (Jordan and Ewing, 1962). Thus, at the experimental pH of 3.7, most ferrocyanide will be present as $[HFe^{II}(CN)_6]^{-3}$. This might cause decreased ferrocyanide adsorption, but it would not cause the decreased ferricyanide adsorption that was observed at pH 3.7. A more likely explanation is that partial dissolution of aluminum hydroxide occurred at low pH conditions. Dissolved Al^{+3} ion concentrations were measured by ICP-MS in control solutions containing poorly ordered Al hydroxide mineral (0.2 g in 20 ml) after 48 h mixing. From this, it was calculated that up to 5.2 wt% Al hydroxide dissolved at pH 3.7, relative to 2.3% at pH 6.2. Thus, dissolution of Al hydroxide at low pH may have resulted in a loss of available mineral surface for iron-cyanide adsorption. Alternatively, Al^{+3} ions released into solution may have formed dissolved complexes with iron-cyanide ions and therefore inhibited iron-cyanide adsorption.

Of the few investigations of iron-cyanide/clay mineral interaction that have been made, these have used iron-cyanide ions as a probe to examine clay interlayer properties rather than examined adsorption directly. In these studies, ferro- and/or ferricyanide was found to be bound within or transported through the interlayers of montmorillonite, kaolinite (Stein and Fitch, 1996) and hydrotalcite (Hansen and Koch, 1994). Although these previous studies noted only low amounts of iron-cyanide adsorption to clay interlayers (and did not provide the pH of the solution), our results showing enhanced adsorption at low pH suggest that electrostatic interaction onto outer clay surfaces may also occur. Therefore, montomorillonite and other clay minerals may play important roles in iron-cyanide mobil-

Table 1
Parameters of Freundlich model fit to Ferrocyanide and Ferricyanide isotherms measured on two model sorbents in the presence and absence of laccase

pH	Parameter	Aluminum hydroxide			Montmorillonite		
		Ferrocyanide		Ferricyanide	Ferrocyanide		Ferricyanide
		(+ Laccase)[a]	(− Laccase)[b]	(− Laccase)	(+ Laccase)	(− Laccase)	(− Laccase)
3.7	K_f	2.261	0.337	0.589	0.235	0.271	0.028
	$1/n$	0.231	1.151	0.614	0.420	0.656	0.875
	R^2	0.774	0.929	0.719	0.837	0.966	0.947
6.2	K_f	5.578	3.022	5.742	0.065	0.021	0.001
	$1/n$	0.788	0.949	2.014	0.590	0.618	1.971
	R^2	0.891	0.976	0.914	0.963	0.654	0.914

[a] In the presence of laccase.
[b] In the absence of laccase.

ity, especially in contaminated soils that are commonly acidic.

At low pH (3.7), ferrocyanide displayed a higher affinity for both minerals compared to ferricyanide, indicating an out-competition of the former for binding sites. This difference, though, not as great as has been observed by others (Cheng et al., 2000), supports the view that ferricyanide will be the more mobile iron-cyanide species at contaminated acidic soil sites.

3.3. Iron-cyanide/enzyme/mineral interaction experiments

Ferrocyanide, laccase, and soil minerals were combined to gauge the likely combined effect of ferrocyanide oxidation by laccase (Fig. 1) and iron-cyanide mineral adsorption (Fig. 2) on iron-cyanide soil mobility. In general, the effect of the presence of laccase on iron-cyanide (initially added as ferrocyanide) adsorption was similar in trend to that expected if all iron-cyanide was converted to ferricyanide (Fig. 3). For example, iron-cyanide adsorption to Al hydroxide at pH 3.7 increased at lower equilibrium concentrations but decreased at higher loadings when laccase was present, compared to absent, much like the trend for ferricyanide versus ferrocyanide. Iron-cyanide adsorption to montmorillonite at pH 3.7 decreased when laccase was present, as would be expected if the ferrocyanide had been converted to ferricyanide. Smaller, but significant, increases in the adsorption of iron-cyanide to both minerals at pH 6.2 were measured with, versus without, laccase were also observed, much like those of ferricyanide versus ferrocyanide adsorption at the same conditions.

These trends suggest that ferrocyanide was oxidized by laccase, even in the presence of soil minerals. That is, it appears that laccase was able to convert ferrocyanide to ferricyanide either prior to or following mineral adsorption. However, iron-cyanide + laccase + mineral system isotherms do not exactly match those of ferricyanide + mineral systems without laccase system, suggesting that ferrocyanide oxidation was not complete. To explain this, a number of chemical phenomena can be considered.

Adsorption of laccase to mineral surfaces may change the effectiveness of laccase in catalyzing ferrocyanide oxidation or it may block iron-cyanide adsorption sites. Laccase adsorption experiments indicate that a large portion of the laccase may be mineral-sorbed under the experimental conditions (as much as 21% and 33% on Al hydroxide and montmorillonite, respectively; Fig. 4). In fact, short-range ordered Al hydroxide has shown a greater affinity for laccase at neutral pH than all other inorganic soil constituents thus far tested (Ahn et al., 2007). For each mineral, the greatest laccase adsorption occurred at the pH closest to each mineral's pznc (closer to net neutral charge). We therefore hypothesize that hydrophobic interaction may be of greatest importance for the adsorption of laccase to these minerals.

While a high degree of enzyme activity retention has been measured for Al oxyhydroxide (Naidja et al., 1997, 2000) and montmorillonite-sorbed (Ruggiero et al., 1989; Naidja et al., 1997, 2000) phenoloxidase enzymes, some suppression of activity will likely occur due to conformational changes. All the previous work on phenoloxidase kinetics, however, has examined activity in terms of phenolic compound degradation and does not necessarily apply to iron-cyanide (and may vary with pH). Further, in these past studies, enzymes were sorbed to minerals prior to substrate introduction. Here, iron-cyanide and enzyme was added separately and simultaneously to solutions containing mineral. If ferrocyanide sorption occurs faster than enzyme adsorption, the chances of contact and enzyme-induced oxidation may be reduced.

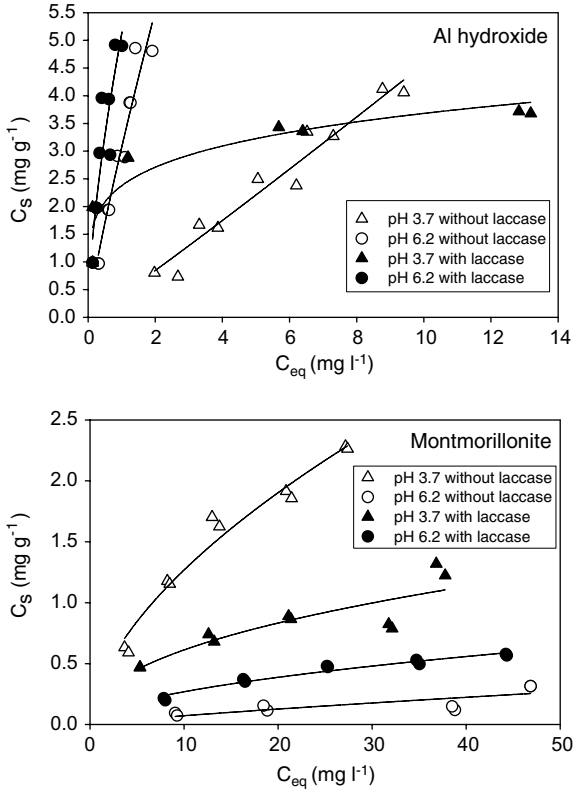

Fig. 3. Adsorption isotherms of iron-cyanide (initially ferrocyanide) on Al hydroxide and montmorillonite at pH 3.7 and 6.2 in the presence and absence of laccase. Freundlich equation isotherm lines are plotted.

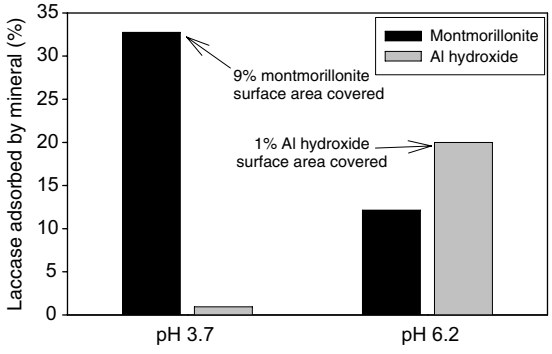

Fig. 4. Laccase adsorption on Al hydroxide and montmorillonite at pH 3.7 and 6.2.

Alternatively, laccase adsorption may have blocked iron-cyanide adsorption sites on each mineral's surface. This is unlikely, however, because the adsorbed laccase can be estimated to have covered only a small portion of each mineral's surface (a maximum of 8.8%, assuming a molecular size for laccase of 15×4 nm, similar to that of albumin: Zimmerman et al., 2004b). It is more likely that the overall charge of each mineral's surface was altered by laccase adsorption. In accordance with its reported pznc of 3.0–3.5 (Claus and Filip, 1988), laccase would have been net negatively charged at all experimental conditions. Adsorbed laccase, therefore, may have altered each mineral's adsorption affinity and adsorption capacity for iron-cyanide, especially in their outer-sphere layer where iron-cyanide complexation likely occurs. In addition, adsorbed proteins are known to exchange charge-compensating cations with clay surfaces such that the resultant mineral surface is more hydrophobic (Quiquampoix et al., 2002).

3.4. Relevancy to soils and soil remediation

The experimental conditions used in this study are relevant to both natural and anthropogenically altered soils. The results demonstrate almost complete enzyme-mediated oxidation of ferrocyanide under a wide range of soil pH's including the acidic conditions commonly found in contaminated soils (pH 2–4: Meeussen et al., 1994; Kjeldsen, 1999). Likewise, the concentrations of iron-cyanide used in these experiments are commonly observed at contaminated groundwater sites (1–50 ppm: Meeussen et al., 1994; Kjeldsen, 1999). One might also ask whether the concentration of enzyme (i.e. laccase) used in these experiments are likely to be found naturally or are reasonable soil amendment quantities for metal contaminant remediation. Although enzyme concentration in soil cannot be determined since isolation and analytical techniques are not yet available, measurements of enzyme activity in soils have been carried out. The results of past soil enzyme studies must be viewed with caution because of the large number of variables that may affect activity rate determination including soil preparation method and the temperature, pH, duration, and type of substrate used for the incubation. The range of laccase or phenol oxidase activities of 10^2–10^6 mol g^{-1} min^{-1} (dry weight soil) have been reported for soils including that of hardwood forests (Criquet et al., 2000; Di Nardo et al., 2004; Boerner et al., 2005), peatlands (Freeman et al., 2004), mosses and lichens (Sedia and Ehrenfeld, 2006) and coastal dune slacks (Van Bodegom et al., 2005). Therefore, even small amounts of soil would likely contain the 1.2 mol ml^{-1} min^{-1} laccase activity used in our experiments and found to fully oxidize all ferrocyanide likely to be present at contaminated sites. Further, soil amendments of small quantities of laccase to severely contaminated soils or anaerobic or waterlogged soils (that have been shown to contain little natural phenol oxidase activity e.g. Pind et al., 1994; McLatchey and Reddy, 1998) should prove feasible and effective.

4. Conclusions

The results presented here demonstrate that soil enzymes such as laccase may mediate the oxidation, and thus enhance the mobility of iron-cyanide in soils. The mobility of iron-cyanide will be most enhanced under low pH conditions, but will be governed by complex relationships between soil minerals and organic matter. While the importance of soil enzymes on organic contaminant speciation is well known, the influence of enzymes on the mobility of inorganic contaminants in soils requires further study to better understand their fate, transport, and associated risk. Even in the mineral-adsorbed state, laccase is able to oxidize a significant portion of the iron-cyanide likely to be present in contaminated soils. Thus, mineral-immobilized laccase may prove effective as a mobilization agent during remediation efforts.

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