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ENHANCED ANAEROBIC TRANSFORMATIONS OF CARBON TETRACHLORIDE BY SOIL ORGANIC MATTER

RICHARD COLLINS and FLYNN PICARDAL*
School of Public and Environmental Affairs, Indiana University, Bloomington, Indiana 47405, USA

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Abstract—Anaerobic, reductive dehalogenation of carbon tetrachloride (CT) by *Shewanella putrefaciens* 200 is enhanced by the presence of a high-organic-carbon soil. In microbial incubations without soil, 29% of the initial 3 ppm (19.5 μM) CT was transformed after 33 h, whereas 64% was transformed after only 18 h when soil was present. In sterile, biomimetic systems using a chemical reductant, 20 mM dithiothreitol, similar results were observed, suggesting that abiotic electron-transfer mediators in the soil were catalyzing the reaction. Destruction of 62% of the soil organic carbon by H_2O_2 resulted in a soil that was less effective in enhancing CT dechlorination. Following separation of the soil organic matter into three humic fractions, the humic acid (HA) fraction catalyzed the dechlorination reaction to a greater extent (270–442 μg of CT per gram of HA) than did the fulvic acid (FA) fraction (149–234 μg of CT per gram of FA), and both were more effective than the fraction containing humin and inorganic minerals (19–26 μg of CT per gram). The results are consistent with a mechanism involving humic functional groups that serve as electron-transfer mediators able to enhance the reductive transformation of CT in the presence of a microbial or chemical reductant. Humic functional group analyses showed that the FA contained more total acidity and carboxylic acidity than did the HA; however, both fractions contained similar amounts of total carbonyl groups and quinone carbonyls. Abiotic, HA-mediated CT transformation in the presence or absence of 20 mM dithiothreitol was found to be pH dependent. At pH 3.6, little CT transformation was observed regardless of whether dithiothreitol was present or not. At circumneutral pH, HA-mediated CT transformation required the presence of dithiothreitol. At pH 8.7, HA-mediated reductive CT transformation occurred both in the absence or presence of dithiothreitol although the transformation was greater in the presence of a reductant. Trichloromethane (chloroform [CF]) production at pH 8.7 was much lower than at circumneutral pH, and volatile organic compounds other than CF were not detected as products in any case.

Keywords—Reductive dechlorination Biodegradation Carbon tetrachloride Humic substances *Shewanella putrefaciens*

INTRODUCTION

As a result of improper disposal, leaking storage tanks, and spillage, carbon tetrachloride (CT) has become a common contaminant of soils and subsurface sediments. It is the fifth most common chlorinated hydrocarbon contaminant of soils and sediments at the U.S. Department of Energy facilities [1]. Because of its relatively high water solubility, high vapor pressure, and low sorption to soil, CT is relatively mobile in the environment.

The anaerobic biodegradation of CT has been shown to occur under sulfate-reducing conditions [2–4], methanogenic conditions [5], denitrifying conditions [6–8], and Fe-reducing conditions [9,10]. Reductive transformations of CT can lead to less toxic transformation products, such as methane [11] and carbon dioxide [6], or transformation products that are of similar or even greater environmental concern than CT, such as chloroform (CF) [9,12]. The latter compound, however, bears fewer halogen substituents and is more susceptible to aerobic biodegradation [13].

With a few exceptions [14,15], experiments examining the microbial reductive dehalogenation of CT have not extensively studied the interactions between microorganisms and soils or sediments. The potential importance of humic substances in the reductive transformation of CT, however, has been studied in abiotic systems. For example, the abiotic reductive dehalogenation of CT by HS^- has been shown to be catalyzed in the presence of soil humic acid (HA) [16]. As HS^- has been shown to reduce the quinone groups in compounds, such as 8-hydroxy-1,4-napthoquinone [17] and HAs [18], Curtis and Reinhard postulated that hydroquinone/quinone moieties within the HAs acted as electron-transfer mediators between the bulk electron donor HS^- and the chlorinated compound CT [16]. In the presence of only the bulk electron donor HS^-, the process was slow. However, in the presence of both HS^- and HA, the reaction proceeded much faster.

Similar results were obtained when 2,6-anthrahydroquinone disulfonate was used as an electron-transfer mediator to catalyze the reductive dehalogenation of hexachloroethane by HS^- [16]. The quinone was used as a model of the more complex quinone groups in soil organic matter (SOM). In addition, other quinone compounds, such as naphthoquinone, have been demonstrated to catalyze the reductive transformation of nitrobenzenes [17], and it is believed that the hydroquinone/quinone couple may dominate the redox properties of humic materials [18,19].

There have been no published reports describing the effects of humic materials on rates of reductive dechlorination reactions in microbial systems. *Shewanella putrefaciens* 200 is a facultative anaerobe previously shown to be capable of dechlorinating CT to CF and unidentified products under anaerobic conditions [9,12]. Previous work with the microorganism *S. putrefaciens* 200 has shown that the rate and extent of CT dechlorination was enhanced by soil [15], suggesting that the

* To whom correspondence may be addressed (picardal@indiana.edu).

soil catalyzed the transfer of electrons from the microorganism to CT. Because certain inorganic minerals have been shown to increase the rate of anaerobic dechlorination reactions [20], it was unclear whether the catalytic factor in this soil was associated with the organic or inorganic fraction. Therefore, the objectives of this investigation were to determine if the catalytic components in the soil were either inorganic or organic, to investigate if a particular humic fraction was primarily responsible for the observed catalytic ability, and to determine if a correlation existed between humic functional group concentrations and catalytic ability in the various humic fractions.

MATERIALS AND METHODS

Microorganism and culture conditions

Shewanella putrefaciens is a gram-negative, non–spore-forming, polarly flagellated rod with an obligate respiratory metabolism [21]. In addition to its preferred electron acceptor, oxygen, it is able to use a variety of alternate electron acceptors under anaerobic conditions, e.g., Fe(III), Mn(IV), NO_3^-, and NO_2^- [22,23]. Although capable of using a wide range of electron acceptors, *S. putrefaciens* is known to use only a limited range of low molecular weight electron donors, e.g., lactate, formate, and H_2, under anaerobic conditions [23]. The strain used in these experiments, *S. putrefaciens* 200, was originally isolated from a Canadian oil pipeline by Obuekwe [24] and has subsequently been shown to be capable of dechlorinating CT to CF and unidentified products under anaerobic conditions [12,25]. The culture was maintained on solid medium of nutrient agar containing 5 g/L of yeast extract (Difco, Detroit, MI, USA) as previously described [12]. Liquid cultures were grown in a 2.5-L, Bioflow 3000 Fermentor (New Brunswick Scientific Company, Edison, NJ, USA), in a medium (hereafter referred to as the Westlake medium) that consisted (per liter) of 2.0 g Na_2SO_4, 0.5 g K_2HPO_4, 1.0 g NH_4Cl, 0.198 g $CaCl_2{\cdot}2H_2O$, 0.1 g $MgSO_4{\cdot}7H_2O$, 19.35 mg $FeCl_3{\cdot}6H_2O$, 0.5 g yeast extract, and 3 ml of 60% (w/v) sodium lactate [26].

Microaerobically grown cells ($p_{O_2} < 1\%$ of saturation in air) were used in all experiments because such cells have been shown to have maximal dechlorination activity [12]. *Shewanella putrefaciens* 200 cells grown in the bioreactor were harvested by centrifugation and resuspended in freshly prepared sterile Westlake medium at 4°C. The density of the suspension was such that an optical density of 1.6 to 2.1 ($\lambda = 600$ nm) resulted when 1 ml of the suspension was added to 9 ml of Westlake medium in dechlorination experiments. Based on preliminary experiments that correlated culture optical density with viable cell counts determined by serial dilution and plating, an optical density of 1.6 corresponds to approximately 4×10^9 cells/ml.

Soil preparation and treatment

The soil was obtained from the banks of a pond in the Indiana University campus in Bloomington, Indiana, USA. Soils and SOM used in all experiments were sterilized before use by γ-irradiation (25 KGy per 50 g of soil) in a ^{137}Cs irradiator. Compared to other sterilization methods, e.g., autoclaving, γ-irradiation has been shown to cause fewer changes in the chemical and physical properties of soil samples and humic fractions [18,27]. The sterility of soil or soil organic matter after irradiation was verified by spread-plating 50 μl of a soil/SOM:Westlake medium slurry on a rich medium (nutrient agar + 5 g/L of yeast extract). In all experiments, no microbial growth was detected after 72 h, indicating that the materials were sterile.

In some experiments, soil was treated with 30% hydrogen peroxide (H_2O_2) to remove SOM. Slurries were made with 20 g of soil and 40 ml of deionized water. Hydrogen peroxide (Mallinckrodt Chemical, Paris, KY, USA) was then added in 5-ml increments with stirring. The oxidation of SOM was considered complete when the soil sample lost its dark color and when conspicuous effervescence ceased [28]. The sample was then heated at 90°C for 45 min to remove excess H_2O_2. This last step was considered essential, as residual H_2O_2 may later affect the redox properties of the soil slurries used in the dehalogenation experiments. During this time, a soil-to-water ratio of approximately 1:2 was maintained by addition of deionized water. Solids were collected by centrifugation and washed with 200 ml of deionized water. Centrifugation and washing were repeated twice additionally before freeze-drying of the treated soil. The amount of soil, treated with H_2O_2, that was added to the 14.6-ml serum bottles was equivalent to the mass that would be found in 2 g of untreated soil [15]. Experimentally, it was found that 17.44 g of H_2O_2-treated soil was recovered from 20 g of untreated soil. Therefore, for both abiotic and biotic dehalogenation experiments involving H_2O_2 treatment, 1.744 g of treated soil was added to the bottles.

Extraction and purification of humic substances

Humic acid and fulvic acids (FAs) were extracted from soil according to the method of Schnitzer [29]. Briefly, 100 ml of anoxic 0.1 N NaOH was added to 10 g of air-dried soil in a 250-ml polyethylene bottle. The procedure was conducted in an anaerobic chamber or while purging the bottle with oxygen-free N_2. After 24 h on a reciprocal shaker table (100–150 rpm), the bottles were centrifuged at 2,600 *g* for 30 min, and the supernatant containing the FA and HA was decanted. The processes of extraction and centrifugation were repeated six times to maximize the recovery of the HA and FA. The efficiency of the extraction procedure was also enhanced by ultrasonically dispersing the soil (140 W for 8 min, Fisher 550 Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA, USA) in 100 ml of deionized water after the third alkaline extraction.

After decanting the alkaline extracts into 250-ml polyethylene bottles, they were immediately acidified to pH < 2 with 2 N HCl [29]. The HAs were then allowed to coagulate for 24 h at room temperature (25 ± 1°C) in the absence of light. The soluble FAs were separated from the coagulated HA at pH < 2 by centrifugation. The soil residue (humin inorganics, and nonextractable macro-organic material) remaining after alkaline extraction (hereafter referred to as humin) was neutralized to the original pH of the soil (pH = 6.15 ± 0.05) using 2 N HCl. The residue was centrifuged, and the supernatant was discarded. To ensure removal of NaCl, the solids were repeatedly (4–5 times) washed with 200 ml of deionized water and collected by centrifugation.

The alkaline extracts resulting from the fractionation procedure described above most likely contain discrete compounds such as simple sugars and amino acids that are coextracted with the humic substances [30]. To remove these low molecular weight compounds as well as NaCl, the HA and FA were purified by ultrafiltration. Because many of the dehalogenation experiments using humic substances were conducted at the pH of the soil (~6.15), the pH of the humic materials

was adjusted to this value with 0.1 N NaOH before ultrafiltration. Ultrafiltration membranes (Spectrum, Laguna Hills, CA, USA) with a molecular weight cutoff of 500 and 10,000 Da were used to purify the FA and HA, respectively. This was done in a 350-ml ultrafiltration cell (Spectrum) using argon gas to pressurize the cell to 60 kPa. The solutions were initially concentrated to 50 ml, diluted with ultrapure water (Millipore, Bedford, MA, USA) were concentrated again. To ensure complete removal of salts and nonhumic substances, this process was repeated four to five times. Samples were then freeze-dried.

Experiments were conducted to determine the humic fraction that was largely responsible for the enhanced CT transformation observed in the experiments of Backhus et al. [15]. In those experiments, CT transformation was examined in the presence of 2 g of soil. In the current experiments, the amount of humic material used was proportional to the mass of the humic material in the soil. For example, because 5.3% of the soil mass was extracted as HA, 5.3% of 2 g, i.e., 0.106 g, was used. Similarly, 0.086 g of FA or 1.8 g of humin was used.

General procedure for dehalogenation experiments

All γ-irradiated soil materials, media, and glassware were degassed for 24 h in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA) before use. Degradation experiments were conducted in 14.6-ml serum bottles that were crimp-sealed with Teflon®-lined, butyl rubber stoppers. These reaction vessels contained 9 ml of Westlake medium and appropriate amounts of soil or humic substances (described earlier). The lactate (18 mM) in the medium served as the sole electron donor in microbial systems because *S. putrefaciens* is unable to use complex natural organic matter for this purpose. Carbon tetrachloride was added to the crimp-sealed bottles by injection of a methanol-based stock solution through the septum. These stock solutions were prepared so that a 5-μl injection of the stock solution would result in an initial aqueous concentration of 3 mg/L before partitioning to solids, assuming Henry's equilibrium and a Henry's constant of 1.244 [31]. The bottles were incubated horizontally at room temperature (25 $\pm$ 1°C), in the absence of light, on a reciprocal shaker table at 100 to 150 rpm for 20 to 24 h to allow for interphase equilibration of CT. Biodegradation experiments were initiated by injection of a concentrated suspension (1.0 ml) of *S. putrefaciens* 200 that would result in a final cell density, after dilution, equivalent to an optical density of 1.6 to 2.1. Control bottles in these microbial experiments were injected with 1.0 ml of sterile medium instead of the cell suspension.

Abiotic degradation experiments were conducted similar to microbial experiments but used 10 ml of Westlake medium that lacked yeast extract or lactate. Instead of addition of a cell suspension, abiotic experiments were initiated by an injection of 200 μl of a filter-sterilized, 1.0 M dithiothreitol (Sigma, St. Louis, MO, USA) stock solution using a N_2-purged syringe. This resulted in a 20 mM dithiothreitol concentration in the 10-ml aqueous volume. Control bottles in abiotic experiments were injected with 200 μl of sterile degassed water instead of the dithiothreitol solution.

An experiment was conducted to examine the effect of pH on the abiotic transformation of CT in the presence of HA. In this experiment, 9 ml of sterile Westlake medium (without yeast extract or sodium lactate) were added to bottles containing 75 mg of HA. To three sets of bottles, 1.0 ml of sterile degassed 0.1 N HCl, deionized water, or 0.1 N NaOH were added to provide a pH of 3.6, 6.2, or 8.7, respectively. Following addition of CT and equilibration, the reaction was again initiated by injection of dithiothreitol to a concentration of 20 mM. Control bottles at different pH values were prepared in a similar fashion but without dithiothreitol. Because the electrode potential of dithiothreitol is pH dependent, additional controls were also prepared with dithiothreitol but without HA. Redox measurements were made in the anaerobic chamber using an Ingold combination electrode (Ag/AgCl reference) (Ingold Electrodes, Wilmington, MA, USA). When conducting such measurements, samples were equilibrated with the electrode for approximately 1 h before recording the electrode potential.

In all experiments, the reaction was terminated by injecting 2 ml of hexane. The serum bottles containing hexane were placed on a rotator for 4 h (100–150 rpm) and then centrifuged at 3,000 rpm for 30 min to obtain a clarified hexane extract for analysis of CT and degradation products.

Analysis of chloro-organic compounds

The CT and CF in hexane extracts were analyzed using a Hewlett-Packard 5890 Series II gas chromatograph equipped with an electron capture detector and a megabore DB-624 column (J & W Scientific, Fulsom, CA, USA). Standards were prepared in hexane to bracket the expected concentrations of CT and CF in the solvent extracts. Standard curves were run before each analysis. Three replicate injections were used to determine the mean mass of CT or CF in each bottle at each sampling point. Our method detection limits allowed us to detect aqueous CT and CF concentrations of 0.45 and 3.8 μg/L, respectively.

Analysis of soil and humic substances

Total organic carbon content of soils and humic substances was performed in a LECO C/S 244 analyzer. Before the analysis of humic substance functional groups, steps were taken to protonate acidic groups. The FAs were purified by ultrafiltration at pH 2. Subsequently, they were washed four to five times in the ultrafiltration chamber with ultrapure (Millipore®) water to remove NaCl and excess acidity. The HAs were precipitated by acidification with 2 N HCl, repeatedly washed with ultrapure water, and collected by centrifugation. Following these steps, the total acidity and carboxylic acidity in humic substances were determined by the barium hydroxide and calcium acetate methods, respectively, as described by Schnitzer [29]. The total carbonyl content in humic substances was analyzed by a redox titration following HA derivatization with hydroxylamine [29]. Quinone carbonyls in humics were quantified by redox titration following reaction with stannous dichloride as described by Schnitzer and Riffaldi [32].

RESULTS AND DISCUSSION

Effects of H_2O_2 treatment on soil catalytic ability

Previous work had demonstrated that a high-organic-carbon soil enhanced the microbial and abiotic dechlorination of CT in batch reactors [15]. To help determine if the enhancement was due to inorganic or organic soil constituents, SOM was oxidized by treatment with H_2O_2. This treatment reduced the total soil organic carbon from 13.13 to 5.03% for an approximate 62% reduction. The effects of removing organic carbon from the soil on the reductive transformation of CT can be seen in Table 1. In some replicate bottles, a chemical reductant

Table 1. Effects of H_2O_2 treatment on the ability of the soil to enhance the abiotic or microbial reductive dechlorination of carbon tetrachloride (CT)[a]

Treatment	CT recovered[b] (%)	CF[c] produced[b] (%)	CT transformed[d] and recovered as CF (%)
(1) Soil controls[e]	90 ± 3	0 ± 0	—
(2) *Shewanella putrefaciens* 200 alone (no soil)	71 ± 3	7 ± 2	24 ± 9
(3) Soil plus *S. putrefaciens* 200	36	5	8
(4) Treated soil plus *S. putrefaciens* 200	53 ± 2	11 ± 2	23 ± 4
(5) 20 mM dithiothreitol alone (no soil)	93 ± 11	0 ± 0	—
(6) Soil plus 20 mM dithiothreitol	7	16	17
(7) Treated soil plus 20 mM dithiothreitol	71 ± 1	4 ± 1	14 ± 4

[a] The mass of untreated soil used in all cases was 2.0 g. The mass of treated soil used was 1.744 g, which was the mass remaining following H2O2 treatment. All data represent values at 18 to 20 h with the exception of those for (1) *S. putrefaciens* alone, and (2) H_2O_2-treated soil plus *S. putrefaciens.* In these cases, bottles were analyzed after 33 h.

[b] Percentage values shown represent the molar percentage recovered following extraction and was based on the initial mass of CT added. Values represent the mean ± SD of two replicates except treatments (3) and (6), which represent one replicate.

[c] CF = chloroform.

[d] Percentage of CT transformed is equal to 100% minus the percentage recovered.

[e] Soil controls contained soil, medium, and CT but lacked bacterial cultures or dithiothreitol.

(20 mM dithiothreitol) was added instead of microbial cultures. Carbon tetrachloride was stable (90% CT recovery and no CF production) in control bottles containing soil but lacking either bacterial cultures or dithiothreitol, and in bottles containing 20 mM dithiothreitol alone (no soil). Even though H_2O_2 treatment only resulted in a 62% reduction of soil organic carbon, this treatment had a marked negative effect on the ability of the soil to enhance CT transformation. In bottles containing cultures of *S. putrefaciens*, 36% of CT was recovered in the untreated soil, whereas 53% remained in the treated soil. The soil apparently retained some catalytic ability following H_2O_2 treatment because CT recovery was less than in cultures of *S. putrefaciens* alone (71% CT recovery). This may have been because of the incomplete oxidation of organic matter during treatment or because of the presence of inorganic materials with catalytic properties.

In bottles containing dithiothreitol, the effect of H_2O_2 treatment was even more pronounced. Only 7% of the added CT was recovered from bottles containing untreated soil as against 71% recovery in bottles containing treated soil. The data clearly show that H_2O_2 treatment reduced the ability of the soil to enhance CT transformation and thereby support the hypothesis that SOM components in the soil are primarily responsible for the observed catalytic effects. Although it is unlikely that inorganic components in an oxic soil, e.g., silicates, oxides, or carbonates, would be chemically modified to a significant extent by H_2O_2 treatment, the effects of such treatment on the inorganic fraction cannot be unequivocally determined without extensive geochemical studies.

In previous studies examining the dechlorination of CT by *S. putrefaciens*, CF production was always nonstoichiometric [12,33]. Consistent with this earlier work, only 24% of the CT transformed by *S. putrefaciens* in the absence of soil was converted to CF in the current studies. Although more CT (64%) was transformed in bottles containing *S. putrefaciens* and untreated soil, less (8%) was recovered as CF. Soil treatment resulted in a higher percentage of the transformed CT being recovered as CF (23%). In none of these cases were either chlorinated methanes or ethanes detected. It is not surprising that the presence of a high-organic-carbon soil reduces the production of CF because the first step of dechlorination is thought to involve a one-electron reduction of CT to form a trichloromethyl radical. This radical could react with organic matter and thereby not be available for subsequent reduction to CF. In previous work studying transformation of ^{14}C-CT in the absence of organic matter, similar nonstoichiometric CF production was explained by radiolabel incorporation into biomass, presumably a result of reactions of the trichloromethyl radical with cell organic matter [12]. Conversion of CT to carbon monoxide, carbon dioxide, or methane was not observed. In the current experiments, humic materials could similarly react with radicals, resulting in decreased CF recovery. The pattern of CF production in the dithiothreitol-containing systems, however, is not consistent with this explanation because similar amounts of the transformed CT were recovered as CF in bottles containing both H_2O_2-treated soil (14 ± 4%) and untreated soil (17%) (Table 1). This suggests that additional pathways for CT transformation may be operative in the abiotic systems.

Effectiveness of the various humic fractions in enhancing anaerobic CT transformation

To determine if the putative catalytic factor in the SOM could be located in a particular humic fraction, we examined the transformation of CT by *S. putrefaciens* in the presence of extracted FA, HA, and humin. Results of these experiments can be seen in Figure 1. The extent of CT transformation was greatest in the presence of the HA fraction (0.6% CT recovery) and the humin fraction (1.0% CT recovery). In comparison, the extent of CT dehalogenation in the bottles containing the FA fraction was much smaller (57% CT recovery). An average of >80% of the CT was recovered from control bottles without *S. putrefaciens* but containing FA, HA, or humin. Chloroform was not produced in any of the control bottles.

A similar dehalogenation experiment was conducted in the absence of *S. putrefaciens* using dithiothreitol as the bulk electron donor. Figure 2 shows the results of this experiment after 18 h. An average of 22, 37, and 73% of the added CT was recovered from the bottles containing the humin, HA, and FA fractions, respectively. We again observed a higher percentage of CF production in dithiothreitol systems compared to bacterial systems. An average of >85% of the CT was recovered

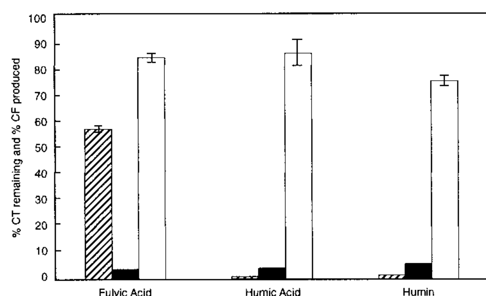

Fig. 1 Anaerobic transformation of carbon tetrachloride (CT) by *S. putrefaciens* 200 in the presence of various humic fractions at pH 6.2 ± 0.1, after 26 h. Carbon tetrachloride remaining in bottles containing *S. putrefaciens* is shown by hatched bars. Chloroform production in these bottles is shown by solid bars. Control bottles (open bars show the remaining CT) were identical to reaction bottles but lacked *S. putrefaciens*. Corresponding to their mass in 2 g of the soil, the mass of the fulvic acid, humic acid, and humin fractions were 0.086, 0.106, and 1.80 g, respectively. The initial aqueous concentration of CT was 3 mg/L. Initial optical density (λ = 600 nm) of the culture was 1.73. Values represent the mean of three bottles, and error bars represent ±1 SD.

from control bottles containing humic material but without reductant. Chloroform was again not produced in control bottles.

It appears then that each of the three humic fractions has the ability to catalyze the abiotic and biotic transformation of CT under anaerobic conditions. This is not surprising because identical functional groups are found in all three fractions. Examined together, however, Figures 1 and 2 show that the FA fraction was less effective in enhancing CT transformation than were the humic acid and humin fractions. However, when the transformation of CT in each fraction is normalized on a mass basis (Table 2), the results indicate that the catalytic capability of each fraction decreases in the following order: HA > FA > humin. The biological and abiotic transformation capacity for each humic component given in Table 2 follows the same order of effectiveness, suggesting that the nature of the initial electron donor is perhaps less important than the chemical characteristics of the humic fraction.

The observed results are consistent with the hypothesis put forth by others [16,34,35] that humic substances contain re-

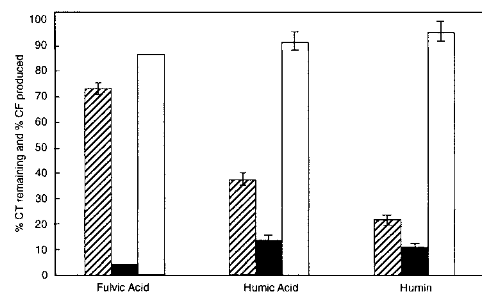

Fig. 2 Anaerobic transformation of carbon tetrachloride (CT) mediated by the different humic fractions in the presence of 20 mM dithiothreitol at pH 6.2 ± 0.1, after 18 h. Carbon tetrachloride remaining in bottles containing dithiothreitol is shown by hatched bars. Chloroform production in these bottles is shown by solid bars. Control bottles (open bars show remaining CT) were identical to reaction bottles but lacked 20 mM dithiothreitol. The mass of the fulvic acid, humic acid, and humin fractions were 0.086, 0.106, and 1.80 g, respectively. The initial aqueous concentration of CT was 3 mg/L. Values represent the mean of three bottles, and error bars represent ±1 SD.

Table 2. Abiotic and microbial carbon tetrachloride (CT) transformation capacities[a] of various humic fractions

Humic fraction	Normalized CT transformation capacity (μg CT per gram of humic material)	
	20 mM dithiothreitol	*S. putrefaciens* 200
Humin	19.8 ± 14.2	26.0 ± 0.0
Fulvic acid	149 ± 11.3	234 ± 7.0
Humic acid	270 ± 15.3	442 ± 0.5

[a] Values represent mean transformation capacities ± SD for the data shown in Figures 1 and 2 and were obtained by dividing the amount of CT transformed by the mass of humic material present. Abiotic and microbial incubations were for 18 and 26 h, respectively.

active components able to serve as electron carriers in reductive transformations. As depicted in Figure 3, *S. putrefaciens* can directly dechlorinate CT at moderate rates [9,12]. If the natural organic matter contains moieties that are relatively quickly reducible by *S. putrefaciens*, and if the reduced moieties are able to rapidly transform CT, enhanced CT transformation as observed in our experiments would result. This hypothesis requires that humic materials be microbially reducible. It has been suggested that some bacteria can use oxidized phenolic compounds and quinones as electron acceptors [36], and recent work has shown that dissimilatory Fe(III)-reducing microorganisms can donate electrons to humic substances [37]. In the latter experiments, the microorganism *Shewanella alga* was able to use humic substances as well as 2,6-anthraquinone disulfonate as a sole electron acceptor for growth. Because *S. alga* is physiologically similar to *S. putrefaciens* 200, it is likely that *S. putrefaciens* 200 also has the ability to reduce humic substances. We cannot rule out the possibility that a portion of the enhancement effect is a result of increased cell numbers or viability resulting from utilization of the humic material as a terminal electron acceptor. Because the abiotic experiments with dithiothreitol show that reduced humic material can rapidly dechlorinate CT, enhanced CT transformation in the presence of reduced humic compounds can clearly occur without a need for microbial growth.

Analysis of major HA and FA functional groups

Because the humin fraction contained residual nonhumic and inorganic materials, we chose the HA and FA fractions for further study. The goal of these additional studies was to determine if common functional group concentrations differed between the HA and FA fractions, and if such differences could account for the greater effectiveness of the HA in catalyzing CT dechlorination. Table 3 shows the concentrations of various oxygen-containing functional groups in the FA and HA fractions. As expected, the FA fraction contained considerably

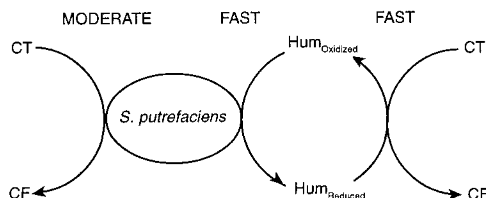

Fig. 3. The hypothetical mechanism describing the enhanced reductive dehalogenation of carbon tetrachloride by *S. putrefaciens* 200 in the presence of humic substances (Hum). Based on the mechanism described by Dunnivant and Schwarzenbach [34].

Table 3. Major oxygen-containing functional group concentrations[a] in humic acid (HA) and fulvic acid (FA) fractions.

Fraction	Total acidity	Carboxylic acidity	Total carbonyl	Quinone carbonyl
FA	9.38 ± 1.00	3.09 ± 0.12	4.34 ± 0.47	0.44 ± 0.16
HA	5.58 ± 0.49	1.57 ± 0.05	3.80 ± 0.42	0.58 ± 0.06

[a] All concentrations expressed as milliequivalents per gram of humic material. All values represent the mean of two or more measurements ±1 SD.

more total acidity and carboxylic acidity. Total carbonyl and quinone carbonyl concentrations, however, were similar.

The results for the total acidity and carboxylic acidity in HA and FA are low when compared to the published values for other humics and fulvics extracted from similar soils [27,38,39]. For example, typical literature values for total acidity range from 5.7 to 8.9 meq/g for HAs and from 8.9 to 14.2 meq/g for FAs. Typical literature values for –COOH groups range from 1.5 to 5.7 meq/g for HAs and 6.1 to 8.5 meq/g for FAs. The values in the literature, however, are for humic material that has been purified with HCl:HF to reduce the ash content. This method also increases the concentration of organic carbon in the HA to approximately 50% [29]. The organic carbon content in the humic material used in our experiments was only approximately 29%. Therefore, the discrepancy between the values in the literature and those here could best be explained by differences in methods used to purify the humic substances.

Quinone concentrations reported in the literature are usually higher in HAs than in FAs [27,38,39]. However, when determining the quinone carbonyl concentrations in the HA, it was extremely difficult to completely dissolve the HA in the reaction solution. Therefore, the method used to determine quinone group concentrations may have underestimated these groups in the HA.

The actual functional groups responsible for enhanced CT transformation cannot be identified by these studies. It has been hypothesized that quinone moieties within humic substances are responsible for catalyzing the reduction of nitrobenzenes and CT [16,17,34]. Although we did not see a direct relationship between quinone concentrations and the increased catalytic ability of the HA fraction, our results do not unequivocally show a lack of quinone involvement. Indeed, our results confirming the presence of quinone moieties in both HA and FA fractions could provide at least a partial explanation for the observed catalytic activities in all fractions. It should also be noted that the redox characteristics of an electron carrying group such as the quinones may be largely determined by its environment in the humic molecule. The molecular environment of the HA could hypothetically result in HA quinones being more effective electron carriers. If so, total quinone measurements, such as those done here, may not give a true picture of the effectiveness of humic substances as electron carriers. In addition, all quinone moieties may not be equally accessible to participate in redox reactions [19].

Although quinones have received much attention, there may be other moieties that also result in an enhancement of dehalogenation reactions. Other possibilities could include reduced sulfur moieties, or complexed trace metals that, in theory, could act similar to biological transition metal complexes. In the first case, Weber et al. recently described the formation of aromatic thiolates resulting from nucleophilic addition of bisulfide to 1,4-benzoquinone [40]. Such compounds might serve as suitable reductants for CT, but formation of such thiolates in our microbial systems is unlikely because no sulfides or sulfur-based nucleophiles (e.g., cysteine) were added, and *S. putrefaciens* does not reduce sulfate. In the latter case, Fe-, Co-, and Ni-containing porphryins have been implicated in catalyzing the reductive dehalogenation of CT and other chloro-organic compounds [12,35,41]. The International Humic Substances Society's standard and reference FA and HA have been shown to contain semiquantitative concentrations greater than 5 μg/g of numerous metals including Fe [42]. We did not attempt to quantify concentrations of transition metals in our studies.

Table 4. pH effects on the abiotic reductive transformation of carbon tetrachloride (CT) in the presence of humic acid (HA)[a]

Treatment[b]	CT recovered[c] (%)	CF[d] produced[c] (%)
pH 3.6, control	95 ± 0.66	0.0 ± 0.00
pH 3.6, dithiothreitol	81 ± 4.1	4.3 ± 0.37
pH 6.2, control	88 ± 2.8	0.0 ± 0.00
pH 6.2, dithiothreitol	21 ± 2.9	17 ± 0.02
pH 8.7, control	11 ± 1.8	2.2 ± 0.23
pH 8.7, dithiothreitol	0.49 ± 0.17	2.9 ± 0.46

[a] All data represent values 20 h after dithiothreitol addition with the exception of pH 8.7 reactors for which values are shown at $t = 10$ h.
[b] All bottles contained HA (75 mg). Control bottles lacked dithiothreitol.
[c] Percentage values shown represent the molar percentage recovered or produced. All values represent the mean of two replicates ± SD.
[d] CF = chloroform.

The effect of pH on the reductive transformation of CT in the presence of HA

Table 4 shows the abiotic transformation of CT in the presence of HA at different pHs values. Almost all (88–95%) of the CT was recovered from control bottles (containing humic material but not dithiothreitol) at pH 3.6 and 6.2. No CF production was observed in these controls. Slight transformation of CT was observed in bottles containing dithiothreitol at pH 3.6 with concomitant production of CF. At pH 6.2, CT transformation in bottles with reductant was extensive, with much more CF production, similar to results shown in Figure 2. In these experiments, CT recovery following the 20- to 24-h equilibration period, but before reaction initiation by addition of dithiothreitol, was almost complete (90–100% CT recovery) in all bottles except at pH 8.7 (data not shown). At pH 8.7, 70 to 77% of the CT was transformed during the equilibration period before dithiothreitol addition (data not shown). Carbon tetrachloride transformation at pH 8.7 was subsequently greater in the presence of dithiothreitol than in its absence as seen by comparison with controls. A very low percentage of the transformed CT at pH 8.7, however, was recovered as CF. No other chlorinated organic compounds, (dichloromethane or chloromethane) were detected and the predominant products at pH 8.7 were not identified. The different product distribution suggests that CT may be transformed by HA at high pH values by a different mechanism than at the other pHs examined. It should be noted that, in the absence of humic material, hydrolysis rates for CT have been shown to be independent of pH (pH = 2–13) [43].

The midpoint electrode potential of dithiothreitol is a function of pH [44], and measured potentials decreased as expected

with increasing pH. In reactors containing HA and dithiothreitol, measured electrode potentials (versus standard hydrogen electrode) were −90 mV (pH 3.6), −180 mV (pH 6.2), and −330 mV (pH 8.7). Similar electrode potentials were measured in bottles containing dithiothreitol but not HA. The lower potential of dithiothreitol at elevated pH values, however, cannot explain increased CT transformation at pH 8.7 because most CT transformation at pH 8.7 occurred before addition of the reductant, and no significant transformation occurred at any of the three pH values in experiments with dithiothreitol but without HAs (data not shown).

The results obtained from this experiment suggest that the HA is a more effective reducing agent at high pH. Schwarzenbach et al. have also reported that the reduction of the nitro group of 4-chloronitrobenzene by two napthoquinone isomers in the presence of H_2S increased with increasing pH [17]. Similar results have been obtained by Curtis and Reinhard, who found that the rate constant for the reductive dehalogenation of C_2Cl_6 by AHQDS at pH 4.5 to be essentially 0 [16]. Between the pH range of 6 and 8 in their experiments, reaction rates increased and the overall reaction was dominated by the semianthrahydroquinone(OH)O^- anion [A(OH)O^-]. As pH was increased from 8 to 10, reaction rates became even higher as the contribution of the 2,6- anthraquinone$(O)_2$ anion [$A(O)_2^{2-}$] became more significant. The effect of pH in our experiments, therefore, might be largely explained if the reactivity of quinones in HA exhibits a similar pH dependence as does AHQDS.

Increased transformation at an elevated pH may also, at least in part, be a result of buffer-catalyzed reactions. An increase in pH increases the concentration of deprotonated forms of the acidic functional groups present in humic substances. Phosphate solutions have been shown to catalyze the transformation of 1,2-dichloroethane in the presence of bisulfide ion [45]. In those experiments, the HPO_4^{2-} ion served as a nucleophile and catalyzed the substitution reaction between 1,2-dichloroethane and H_2O. Such buffer catalysis has also been proposed to explain increased CT dehalogenation by *S. putrefaciens* 200 at elevated phosphate concentrations [10].

CONCLUSIONS

A complete understanding of reductive transformations of pollutants in natural sediments must include knowledge about microbial-pollutant interactions with natural organic matter. By removing ∼62% of the organic carbon in the soil used in the current studies, a significant decrease in the rate of anaerobic CT reductive dehalogenation was observed, regardless of whether reducing power was provided by microbial metabolism or addition of a chemical reductant. The hypothesis that the catalytic factor was associated with the organic fraction was verified by experiments showing that all three humic fractions catalyzed the biotic and abiotic reductive dehalogenation of CT. On a mass basis, the HA fraction was a more effective catalyst than the FA fraction.

The data in Table 2 suggest that the extent of enhancement is characteristic of a particular humic fraction, regardless of whether the ultimate reductant is microbial or chemical in nature. This is consistent with a mechanism involving electron-transfer mediators in the humic material. Further experiments are needed to better identify the moieties acting as these electron carriers.

The results indicate that rates of microbially mediated transformation of some xenobiotic compounds in the environment may be more rapid than those calculated using laboratory systems devoid of natural organic matter. The data also suggest that compounds such as CT may be indirectly transformed by bacteria unable to directly degrade the xenobiotic, but able to reduce humic electron carriers capable of reducing the xenobiotic. Additional research is needed to identify the range of xenobiotics able to be transformed by reduced humic material and to determine if transformation rates in low-organic, native sediments and soils can be increased by addition of humic materials.

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