Environ. Sci. Technol. **2004**, *38,* 783−789

Cl K-edge X-ray Spectroscopic Investigation of Enzymatic Formation of Organochlorines in Weathering Plant Material

RACHEL G. REINA,[†] ALESSANDRA C. LERI,[‡] AND SATISH C. B. MYNENI*[,†,‡,§,∥]

Department of Civil & Environmental Engineering, Department of Chemistry, and Department of Geosciences, Princeton University, Princeton, New Jersey 08544, and Earth Sciences Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, California 94720

The contribution of halocarbons from plant weathering to the total organohalogen budget of terrestrial systems is gaining recognition. To evaluate the formation of such halocarbons, speciation of chlorine in *Sequoia sempervirens* (redwood) needles was examined in the presence of an external chloroperoxidase (CPO) enzyme using Cl K-edge X-ray absorption spectroscopy. The Cl forms in fresh and naturally weathered needles and in model laboratory reactions were compared. To provide a straightforward analogue to the enzymatic chlorination in plants, chlorination reactions were conducted for phenol, a common moiety of plant macromolecules. Plant material chlorination was also examined in the presence of hypochlorite in an ancillary mechanistic investigation. The dominant form of Cl in fresh, unreacted plant material was found to be inorganic Cl^-, which was partially converted to organochlorine in the presence of CPO. Chlorination is affected by the nature of reactant (CPO, H_2O_2) addition, reaction time, and temperature. The organochlorines produced in these laboratory investigations closely resemble those produced during the natural weathering of redwood needles. A striking consistency in chlorine speciation observed among the various sample types suggests that (i) CPO produced by terrestrial organisms could play a vital role in the generation of organochlorines associated with the degradation of plant material and (ii) initial targets of enzymatic chlorination might include lignin-like macromolecules rich in aromatic character and hydroxyl groups. These findings lend further credibility to a significant biogenic contribution to the global organohalogen burden by elucidating a probable route of enzymatic chlorination of natural organic matter in terrestrial systems.

Introduction

Although anthropogenic sources are currently considered the primary origin of organohalogens in the environment, studies conducted in the last several years have indicated a major natural source of these products (*1, 2*). One feasible source of natural organochlorines in soil systems is the chlorination of plant material during weathering. Unexpectedly high, temporally variable concentrations of organohalogens in decaying forest leaf litter and in soils remote from anthropogenic sources indicate that both halogenation and dehalogenation are related to natural organic matter decomposition (*3*). Fresh plant matter has been analyzed by Nkusi and Muller (*4*) and Flodin et al. (*5*), with quite disparate results. Nkusi and Muller (*4*) determined that up to 75% of halogens in one terrestrial plant species (*Euphorbia charasias*) were present as organohalogens, while Flodin et al. found a maximum of 10% organohalogens in fresh spruce wood (*Picea abies*) and less in fresh sphagnum moss and birch leaves. More recently, in-situ X-ray absorption spectroscopy (XAS) studies have indicated that the concentration of chloride (Cl^-), the dominant form of chlorine in fresh plant material of several plant species, appears to decrease while organochlorine concentration increases as the plant material weathers naturally in the environment (*6*). However, organic molecule chlorination reactions in weathering plant materials remain poorly understood.

Researchers have examined chlorination reactions of various organic molecules in the presence of hydrogen peroxide (H_2O_2), Cl^-, and chloroperoxidase (CPO), an Fe-heme-based fungal enzyme. The model organic substrates used in these studies included simple organic molecules (such as phenols, cresols, and benzoic acids; *7*−*10*) and macromolecular weathering residues of biological material (such as humic acid (HA) and fulvic acid (FA)) isolated from various natural sources (*11*−*14*). CPO-catalyzed reactions of substituted and unsubstituted phenols with H_2O_2 and Cl^- in the pH range of 2.0−2.75 produced *o*- and *p*-chlorophenols as the dominant fraction, and the yields ranged from trace concentrations to 25% depending on the degree of chlorination of starting material (*10*). Although dichlorination of phenols did not occur even in the presence of excess enzyme (*10*), dichlorination was reported in the case of benzoic acids with very small yields of the product (*15*). Previous investigations also showed the formation of aliphatic chlorinated compounds at trace concentrations (*14*).

To date, all isolated humic substances (HA and FA) examined using XAS have been found to contain organochlorines (*6*). The concentration of organochlorines increased as much as 30-fold upon enzymatic chlorination of humic substances (*11*). While this reaction has been examined in the laboratory using CPO isolated from *Caldariomyces fumago*, additional studies have also revealed a widespread capacity for organochlorine production among other filamentous fungi, the Basidiomycetes, a group of fungi known to be responsible for lignin degradation (*16*−*18*). Chlorination assays of soils have revealed widespread occurrence of an enzyme exhibiting haloperoxidase activity (*19*). However, the potential interactions of fungal enzymes with plant material and their influence on halogenation of organic matter were not examined.

Here, we show that organochlorines are produced from reactions of Cl^- in plant material in the presence of a CPO enzyme released by common fungi in the environment. We also examined reactions of Cl^- with phenol in the presence of CPO, since phenolic groups are important building blocks of unweathered macromolecules in plants (*20*) and because chlorinated phenols constitute the dominant fraction of halogenated organic molecules in weathered plant material and humic substances (*6*). The chlorination of plant material

* Corresponding author phone: (609)258-5848; e-mail: smyneni@princeton.edu.

[†] Department of Civil & Environmental Engineering, Princeton University.

[‡] Department of Chemistry, Princeton University.

[§] Department of Geosciences, Princeton University.

[∥] Lawrence Berkeley National Laboratory.

10.1021/es0347336 CCC: $27.50
Published on Web 12/23/2003

was tested as a function of concentration of CPO and H_2O_2, reaction time, and temperature. The feasibility of a widely accepted but not undisputed mechanism (*9*, *21*), involving hypochlorous acid as a reactive intermediate in CPO-mediated halogenation reactions (*22*), is also addressed. XAS was used to explore the reaction products of an established CPO-mediated procedure with phenol and the novel CPO-induced organohalogenation in weathering plant material. This technique allowed the direct examination of reaction products without subjecting the sample to isolation or special preparation procedures. Furthermore, comparisons of CPO-reacted plant materials with naturally weathered plant materials can be made using XAS.

Experimental Section

Materials. The plant material used in this study was collected from Big Basin Redwoods State Park, California. Fresh green needles from healthy redwood trees (*Sequoia sempervirens*) and senescent and weathered needles from soil surfaces in their vicinity were collected in zipper-sealed plastic bags and stored at 4 °C prior to treatment and analysis. The fresh green needles were gently rinsed with deionized water, and excess moisture was removed prior to the reactions with enzyme. Fresh plant needles were preferred over the senescent needles for this investigation because the XAS of fresh needles showed only inorganic chloride whereas the XAS of senescent needles exhibited organohalogens, whose concentration changed significantly from sample to sample (*6*). The heterogeneity in Cl speciation of senescent needles interferes with the estimation of organohalogens formed by the enzymatic reactions. In addition, macromolecules (e.g., lignins, polyphenols, and pectins) present in fresh and senescent leaves are considered to be similar. Although variations are expected for carbohydrates, which tend to degrade rapidly, the published results do not indicate their involvement in halogenation.

Chloroperoxidase, isolated from the fungi *C. fumago*, was purchased from Sigma (St. Louis, MO). When required, the original CPO-containing solution (enzyme concentration ~12 500 units/mL, pH buffered by phosphate solution around 4.0) was diluted with phosphate solutions buffered at pH ~3.7 (± 0.3). We selected this pH because enzyme reactivity is greatest in this pH range, weathering of plant material produces a pH close to 4.0, and the pH of all surface soils (O horizons) is close to 4.0. Reagent-grade phenol (>99.5% pure), H_2O_2 (30%), and sodium hypochlorite solution (available chlorine ≥4%) used in this study were also obtained from Sigma.

Chloroperoxidase Reactions with Phenol. Chlorination of phenol was examined in the presence of NaCl, H_2O_2, and CPO. A solution containing phenol (25 mM) and NaCl (25 mM) was prepared, and its pH was buffered at 3.5 by phosphate solutions (prepared from H_3PO_4 and NaH_2PO_4 in deionized water). About 0.5 mL of a 0.3% H_2O_2 solution and ~125 units of CPO were added in rapid succession to the above solution. The solution color immediately changed from colorless to pale brown. The mixture was stirred for 30 min, at which point a second equivalent of dilute H_2O_2 (0.3%) was added. After 2 d of reaction, a pale rust-colored precipitate had accumulated at the bottom of the flask. This solid was isolated by centrifugation, rinsed 4−5 times with deionized water to remove residual Cl^-, and smeared on organochlorine-free cellophane tape for XAS analysis.

Chloroperoxidase Reactions with Plant Material. Green needles and their pulp were used to examine chlorine transformations at room temperature (22 ± 3 °C). Needles were pulped, using a ceramic mortar and pestle, to accelerate chlorine transformations because intact redwood needles reacted too slowly to observe chlorine transformations in the time frame of these experiments (details discussed later).

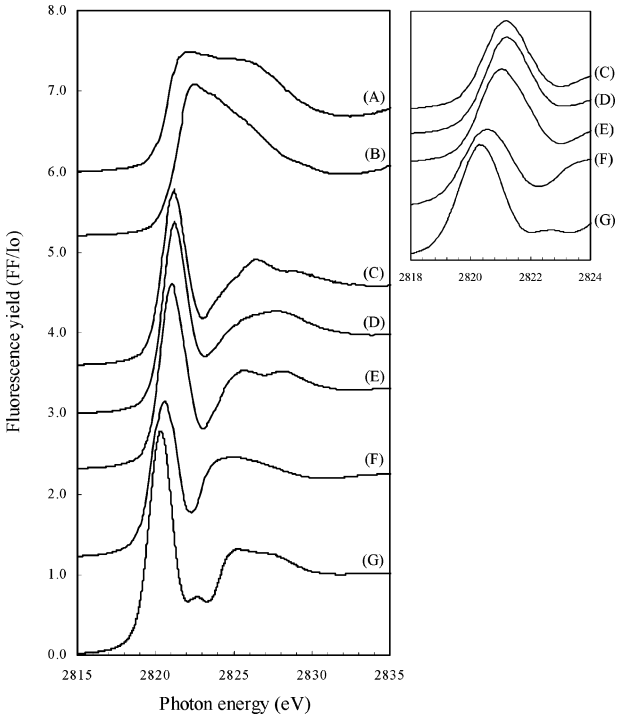

FIGURE 1. Cl K-edge NEXAFS spectra of Cl in different coordination environments in inorganic and organic compounds. (A) Aqueous Cl^-, (B) solid glycine hydrochloride, (C) 2-chlorobenzoic acid, (D) chlorophenol red, (E) tetrachlorophenol, (F) chlorodecane, and (G) chloroform. The inset shows near-edge features.

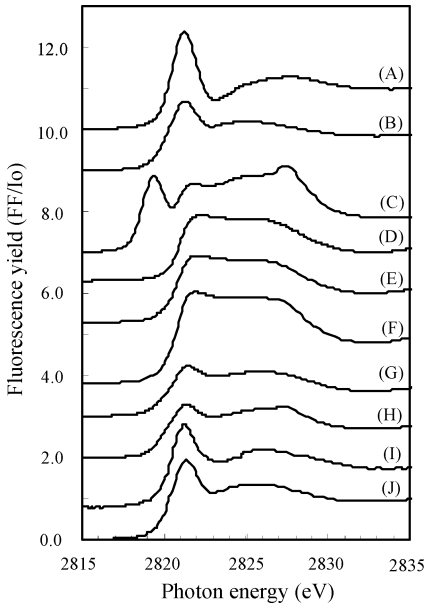

FIGURE 2. Cl K-edge NEXAFS spectra of plant needles reacted with CPO and HOCl. Spectra of phenol reacted with Cl^- and CPO, and pure Cl-containing compounds are also shown for a comparison. (A) Clorophenol red, (B) phenol + CPO + NaCl + H_2O_2, (C) NaOCl-(aq), (D) aqueous Cl^-, (E) unpulped redwood needles + 5 mM NaOCl after 2 d, (F) needle pulp + 5 mM NaOCl after 10 min, (G) needle pulp + 5 mM NaOCl after 2 d, (H) needle pulp + 50 mM NaOCl after 2 d, (I) needle pulp + CPO + H_2O_2 (short-term, multiple addition sample), (J) weathered needles attached to tree.

Pulping does not cause any chlorine transformations in the absence of enzyme (Figures 1 and 3A).

Reactions of CPO with freshly prepared plant needle pulp were conducted over a few hours to several days. For short-term experiments (<9 h), the pulp was reacted with 500 u

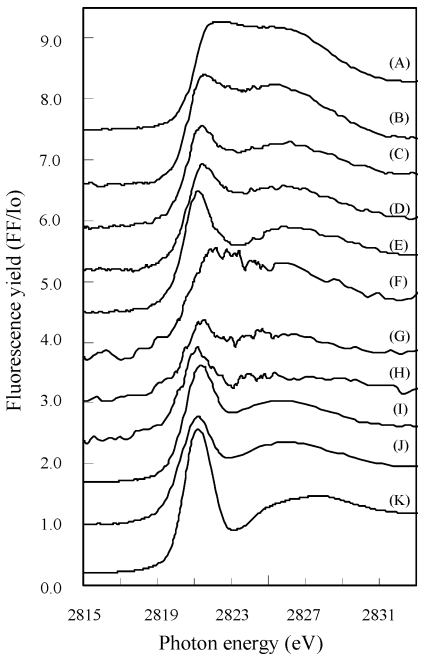

FIGURE 3. Cl K-edge NEXAFS spectra and Cl speciation of CPO-reacted and naturally aged redwood needles. OC: organochlorine. (A) Plain needle pulp (no reactants) exposed to X-ray beam for 100 min (100% Cl^-); (B) pulp sample reacted with dilute CPO and H_2O_2, reaction time 0.33 h (86% Cl^-, 14% OC); (C) pulp sample reacted with dilute CPO and H_2O_2, reaction time 1.33 h (68% Cl^-, 32% OC); (D) multiple addition pulp sample, reaction time 9 h (67% Cl^-, 33% OC); (E) multiple addition pulp sample, reaction time 4 h (35% Cl^-, 65% OC); (F) long-term pulp sample, no reactants added (100% Cl^-); (G) long-term pulp sample, CPO only (51% Cl^-, 49% OC); (H) long-term pulp sample, CPO + H_2O_2 added (29% Cl^-, 71% OC); (I) naturally weathered reddish brown redwood needles attached to tree (40% Cl^-, 60% OC); (J) naturally weathered redwood needles collected from soil surface (31% Cl^-, 69% OC); (K) chlorophenol red.

of CPO and 200 μmol of H_2O_2, which were added according to one of two different protocols: (a) entirely at once (single addition method), and (b) in four increments (125 u of CPO and 50 μmol of H_2O_2 for each addition) distributed equally over the course of several hours (multiple addition method). Pulp was also reacted with dilute reactants for concentrations of 125 u of CPO and 50 μmol of H_2O_2. For XAS analysis, the enzyme-reacted plant material was loaded into the 1-cm^3 hollow aperture of a sample cell, the window of which was made of X-ray transparent, Cl-free polyfilm. The sample size was approximately 0.7 g. The sample cells were made with Cl-free PTFE or plastic.

In longer-term experiments (reaction times of 3−7 d), redwood needle pulp (about 0.85 g/sample) was placed in 15-mL polypropylene centrifuge tubes with 125 u of CPO at the beginning of the reaction period. Hydrogen peroxide was then added in 14 5-μmol increments over 77 h. Samples omitting one or both reactants were also prepared to test the nature of Cl transformation in the absence of the enzyme and oxidant. Deionized water was added to samples not containing H_2O_2 to maintain consistency in the amount of liquid (approximately 8 mL) added to the samples.

To examine the effect of elevated temperature on the progress of the reaction, samples were prepared and maintained at 43 (± 2) and 71 (± 4) °C, in addition to those examined at room temperature (22 ± 3 °C). Redwood needle pulp and H_2O_2 were placed in separate 15-mL polypropylene centrifuge tubes and equilibrated in a water bath at the appropriate temperature for approximately 3 h, then combined with CPO, and returned to the bath for approximately 4 h reaction time. Intact needles were also prepared similarly at each temperature. Freshly collected plant needles were also subjected to a 24-h oven-drying at 50 °C to determine whether drying alone might engender production of organochlorines.

OCl^- Reactions with Plant Material. Redwood needle pulp was reacted with unbuffered 5 and 50 mM hypochlorite solution in separate centrifuge tubes at room temperature (~25 °C). The tubes were sealed for 2 d before the plant pulp was removed from solution, rinsed thoroughly with deionized water to remove unreacted OCl^-, and mounted on a sample holder for XAS analysis.

XAS Data Collection and Analysis. The coordination environment of Cl in natural and enzyme-reacted samples was identified directly using XAS, specifically near edge X-ray absorption fine structure (NEXAFS) spectroscopy at the Cl K-absorption edge. The spectral features at the Cl K-edge correspond to electronic transitions from the Cl 1s core orbitals to vacant atomic and molecular orbitals of 3p character. Chlorine oxidation state modifies the energy position of the absorption edge, which shifts to higher energy as the oxidation state of Cl increases. Between −I and +VII oxidation states of Cl, there is an almost 10 eV difference in the energies of absorption maxima, with intermediate Cl oxidation states exhibiting near-edge transitions at values in between (*6*). Compared with inorganic chloride compounds, the absorption maxima of which occur around 2822.3 eV, C−Cl bonds exhibit intense features in the range of 1−2 eV lower (Table 1, Figure 1). The intense spectral features in organochlorine compounds correspond to the 1s → π^* and σ^* molecular orbital transitions. In addition, monochlorinated aliphatic compounds display absorption maxima at ~0.6 eV lower energy than Cl connected to aromatic carbon. Using this information along with more subtle characteristic differences in postedge oscillations, the distinction between inorganic, aliphatic, and aromatic chlorine becomes manifest and can be quantified using least-squares fitting with model compounds (Table 1).

Although absolute Cl concentrations can be obtained from the X-ray spectra using a series of Cl standards (with identical matrix and bulk density to that of samples), concentration estimates are not attempted in this investigation. This is because of the difficulties associated with the preparation of Cl standards with plant pulp and other reactant mixtures and with the packing of plant pulp uniformly to obtain approximately the same bulk density for all samples and standards. However, we obtained the fractions of different Cl species using the procedures discussed above.

The NEXAFS spectra were collected at the Stanford Synchrotron Radiation Laboratory, using beamline 6-2 and Si(111) monochromator crystals. A He flight path chamber was used to enhance the incident photon flux on samples while preventing interference with the Cl signal from ambient Ar. The NEXAFS spectra were collected using the sample fluorescence, measured with a Lytle detector. The slits in front of the monochromator crystals were set close to 1 × 10 mm. The spectra were collected with a step size of 0.08 eV at the Cl absorption edge and about 0.3−1.0 eV above and below the edge and calibrated with respect to the most intense low-energy transition in KCl at 2822.8 eV. EXAFSPAK (version 2.7) was used to calibrate spectra, average multiple scans where appropriate, and format files for further processing.

Cl speciation of reacted samples was conducted using nonlinear least-squares fitting of sample spectra with the spectra of compounds of known structure (model compounds). WinXAS (version 2.0) was employed for this part of the spectral analysis. The NEXAFS spectra of model compounds had previously been collected (see ref *6*) and included saturated and unsaturated, cyclic, aromatic, acidic, and hydrogen-bonded organochlorine compounds as well as various inorganic compounds and chloride salts. The pro-

TABLE 1. Cl 1s NEXAFS Features of Organic and Inorganic Molecules

Cl coordination environment	compound	absorption maximum (eV)[a]	postedge features[b]
aromatic C−Cl[c]	chlorophenol red	2821.2(s)	2825.3(b), 2827.7(b)
	tetrachlorophenol	2821.1(s)	2825.2(s), 2827.9(b)
	2-chlorobenzoic acid	2821.1(s)	2824.4(b), 2826.3(s), 2828.8(b)
	3-chlorobenzoic acid	2821.1(s)	2824.2(b), 2826.7(s), 2829.5(b)
aliphatic C−Cl[c]	chlorodecane	2820.5(s)	2823.7(s), 2825.7(b)
	chloromethane	2820.5(s)	2825.1(b), 2827.2(b)
	chloroform	2820.3(s)	2822.5(s), 2824.4(b), 2827.3(b)
	trichloroacetic acid	2820.2(s)	2822.4(s), 2825.1(s), 2826.9(b)
	carbon tetrachloride[d]	2819.9(s)	
cyclic C−Cl[c]	monochlorodimedone	2821.2(s)	2824.0(s), 2825.4(s), 2827.5(s)
organic hydrochloride[c] (H-bonded Cl^-)	trizmahydrochloride	2822.4(s)	2824.9(s), 2827.0(s), 2832.0(b)
	glycine hydrochloride	2822.1(s)	2824.0(s), 2825.8(s), 2826.0(b)
inorganic Cl^- (aq)[e]	HCl, NaCl	2822.2	2825.5, 2843.3
	$CaCl_2$	2822.2	2825.1, 2842.4
	$BaCl_2$	2822.4	2825.3, 2842.4
	$FeCl_3$	2821.9	shoulder: 2820.1; 2824.8, 2828.0
	$Ca(OCl)_2$	2819.1(s)[f]	
inorganic Cl^- (s)[e]	KCl	2822.6	2826.4, 2828.3, 2832.7, 2835.2, 2836.8, 2838.1, 2842.0
	$FeCl_3 \cdot 6H_2O$[g]	2823.5	preedge: 2817.2(s); 2825.0, 2839.3

[a] Error in absorption maximum assignation: ±0.1 eV. [b] Error in assignation of postedge features: ±0.3 eV due to broadness of peaks. [c] Peak energies determined by fitting spectra up to 15 eV above the edge: (b) = broad; (s) = sharp. [d] From ref *40*. Value recalibrated to our models. Larger uncertainty is expected according to errors (±0.2 eV) quoted in ref *40*. [e] Values assigned from observed spectral maxima. [f] This peak does not correspond to the expected absorption maximum for a "Cl^+" species and appears at low energy probably due to partial Π character of the O−Cl bond. Higher energy features are not reported because they appear to be obscured by artifacts of oxidation or other conversion processes causing spectral features to change over time. [g] For additional X-ray absorption information on metal chloride compounds at the Cl K-edge, refer to refs *41* and *42*.

pinquity of the Cl-edge NEXAFS spectra of aliphatic and aromatic Cl compounds results in up to 8% projected error in Cl speciation.

Sample alterations in the presence of the X-ray beam were also studied in detail. Our studies suggest that plant needles and their pulp and H_2O_2-reacted plant samples do not exhibit changes in the presence of beam even after a continuous exposure of 12 h. However, the CPO-reacted plant needle pulp showed faster chlorination in the presence of beam and exhibited significant alterations over a period of 1 h. Hence fast scans were collected with a total beam exposure of <0.08 h for all CPO-reacted samples. Organic molecule halogenation induced by the X-ray beam in this time is negligible relative to the reactant-induced halogenation that occurred in the absence of beam exposure.

Results

Chlorine Speciation Using NEXAFS Spectroscopy. Cl K-edge NEXAFS spectra allow the chemical state of chlorine in unprocessed natural samples to be probed directly. Previous studies have indicated that the Cl absorption edge is sensitive to the coordination state and bonding environment around Cl and that the absorption edges of Cl for inorganic Cl^-, aromatic Cl, and aliphatic Cl are present at 2822.3, 2821.1 (± 0.1), and 2820.2 (± 0.3) eV, respectively (Table 1, Figure 1; *6*). Furthermore, the absorption edge of aliphatic Cl is shifted to lower energy with an increase in the degree of chlorination of the molecule (e.g., di-, tri- and tetrachloro compounds). Although the absorption edge of chlorinated aromatics—for example, chlorophenols—is not affected by the extent of Cl substitution, the near-edge features a few electronvolts above the absorption edge are different for various mono- and polychlorinated phenols. Similarly, chlorinated phenols and benzoic acids exhibit different spectral features a few electronvolts above the main absorption edge. Thus, the absorption edge energies and the fine structure in the Cl-NEXAFS spectra of heterogeneous natural samples can be used in the identification of aliphatic, aromatic, and inorganic Cl species (Table 1). Coordination states and fractions of all Cl forms can be determined using XAS, which is also the only available spectroscopic technique for obtaining in-situ information on the dominant forms of Cl and their relative abundance in organic macromolecules associated with the solid phase. These characteristics make XAS an ideal technique for examining chlorine chemistry in heterogeneous natural samples.

Chlorination of Phenol in the Presence of CPO. Cl-NEXAFS spectra of phenol reacted with Cl^- in the presence of CPO indicate a significant conversion of Cl^- to organochlorine after 2 d of reaction (Figure 2B). Pale rust-colored precipitate formed immediately after these reactants were added, and the isolated floc from this mixture displayed about 35% aromatic Cl, 18% aliphatic Cl, and the rest in the form of unreacted Cl^-. Wannstedt et al. reported the formation of *o*- and *p*-chlorophenols in these reaction mixtures (*10*). However, our studies indicate the presence of a significant fraction of aliphatic compounds in addition to the aromatic compounds. The facility of enzymatic chlorination of aromatics suggests that phenolic groups present in lignins and polyphenols of plant needles may convert to chlorophenols in the presence of CPO.

Chlorination of Plant Material in the Presence of Reaction Intermediate HOCl. Analysis of the needle pulp reacted with 5 mM HOCl for 2 d revealed formation of 24% aromatic and 9% aliphatic chlorine, with 67% of the total chlorine remaining as Cl^- (Figure 2G). The coordination environment of inorganic Cl^- could not be identified in this sample because its spectral features did not match any of the known Cl^- spectra. Reaction at higher HOCl concentration (50 mM) resulted in a comparatively greater content of chlorinated hydrocarbons in plant pulp, with a breakdown of 55% Cl^-, 20% aromatic, and 25% aliphatic (Figure 2H). However, additions of 5 mM HOCl to intact needles resulted in a mere 2% conversion of total chlorine to organochlorine after 2 d (Figure 2E).

Chlorination of Plant Material in the Presence of CPO. The NEXAFS spectrum of Cl in unreacted plant pulp exhibits a distinct peak at ~2822.3 eV. This suggests that Cl in fresh plant material is present in the form of inorganic, hydrated Cl^- as the primary Cl species without any detectable

organochlorine (Figure 3A). Inorganic Cl^- in plant samples may also be present in a H-bonded form, which is similar to that of hydrated Cl^- but exhibits H-bonding interactions with organic molecule functional groups. Such variations in H-bonding interactions modify the electronic transitions of Cl and affect the energy of the Cl absorption edge (Figure 1B). As discussed below, extensive H-bonding is characteristic of dried redwood needles. However, in naturally weathered needles and those reacted with CPO and H_2O_2, Cl^- converts to organochlorine, causing the edge to shift to low energy (~2821 eV). In addition, the intensity of this feature increases with the progress of the CPO-mediated reaction.

Chlorination of plant material is affected by the nature of reactant addition and the reaction time. The short-term pulp samples (<9 h) to which reactants were added in a single addition showed markedly different Cl transformation from that observed in the multiple addition samples (Figure 3). Single addition samples prepared with dilute H_2O_2 (50 μmol) and CPO (125 u) produced about 14% and 32% organochlorine for reaction times of 0.33 and 1.33 h, respectively (Figure 3B,C). Aromatic organochlorine is the primary product formed in these reactions. However, transformation of Cl^- was absent when H_2O_2 was added at high concentration (~200 μmol) to the mixture of pulp and enzyme (spectrum not shown). This is possibly a result of interactions between the reactants or between reactants and reaction intermediates. The failure of CPO to halogenate organic substrates has been attributed by previous researchers to the inactivation of CPO by HOCl, a putative reaction intermediate that may chlorinate the heme portion of the enzyme when its concentration builds to excess (*23*). This limitation has been circumvented (*11*−*13*) by the addition of H_2O_2 in small amounts. We applied this sample preparation protocol in both our short-term multiple addition samples and our long-term samples, all of which exhibited organic halogenation.

The short-term multiple addition samples (four doses of 125 u of CPO + 50 μmol of H_2O_2) exhibited up to 65% of total Cl converted to organochlorine within 4 h (Figure 3E). The fractions of aromatic and aliphatic organochlorine are about 55% and 10% of the total Cl, respectively. This high degree of Cl conversion was noted in the case where the reactants (H_2O_2 and CPO) were added separately to the plant needle pulp. However, when H_2O_2 and CPO were mixed prior to mixing with pulp, a much lower conversion of Cl^- was recorded (Figure 3D), no aliphatic Cl was detected, and the product Cl speciation is identical to the single addition sample reacted for 1.33 h (Figure 3C, discussed above). Although the local coordination environment of the aliphatic Cl identified in several CPO-reacted samples is best represented with chlorodecane or chloromethane forms, the aliphatic forms present could be of larger molecular size and could contain other functional groups.

When samples were reacted for 7 d with multiple additions of H_2O_2, percentages of organochlorines observed were higher than those seen in short-term samples. NEXAFS spectroscopy studies indicate that organochlorine formation is absent in samples without reactants (Figure 3F). The CPO-reacted samples, however, contain significant amounts of organochlorine: 71% of the total chlorine in the presence of added peroxide (Figure 3H) and 49% in the absence of added peroxide (Figure 3G). The compositions of these samples were respectively 29% Cl^-, 46% aromatic organochlorine, and 25% aliphatic organochlorine and 51% Cl^-, 34% aromatic organochlorine, and 15% aliphatic organochlorine. The ratio of aromatic to aliphatic chlorine is approximately 2:1 in these samples. At this time, absolute comparisons of Cl transformation extent between short-term and longer-term studies cannot be made. Concentrations of chlorine species, as estimated by raw spectral fluorescence intensity, in the 1-week samples were approximately one-fifth of those observed in the several-hour samples. This discrepancy is very likely due to leaching of inorganic and, perhaps to a lesser extent, organic chlorine species from the plant sample into the surrounding aqueous phase; it may also be explained by the formation and release of volatile organochlorines. These eventualities underscore the importance of incorporating mass balance calculations into future investigations of Cl transformations in natural samples.

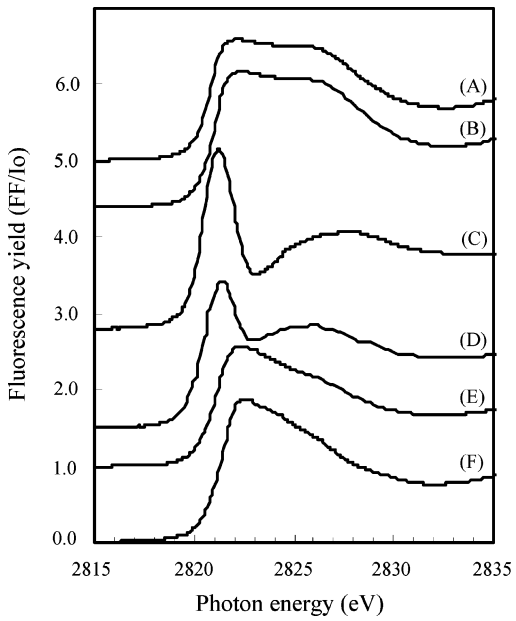

FIGURE 4. Effect of drying on chlorine speciation in fresh redwood needles. (A) Aqueous Cl^-, (B) unreacted redwood needles, (C) chlorophenol red, (D) naturally weathered needles, (E) oven-dried needles, (F) glycine hydrochloride.

The reaction of CPO and H_2O_2 with unpulped redwood needles suggested that longer reaction times are necessary for the formation of organochlorines in needles with intact cuticle. Enzyme-mediated transformations were not observed in any of the intact samples that were reacted for <9 h. However, a sample reacted for 1 yr with a small portion of the reacting solution (170 u of CPO + 6.5 μmol of H_2O_2, reactants added at the beginning of the reaction only) showed a significant organochlorine fraction (~50% of the total chlorine). This sample initially was bathed in reactant solution but dried a few weeks later because of the absence of new reactant doses. Chlorination of organic molecules would have continued further in this sample if more reactants had been supplied. Possible reasons for and implications of this finding are discussed below.

Temperature Effects. The influence of temperature on the enzymatic halogenation of plant needle pulp was examined at 43(± 2) and 71(± 4) °C, in addition to those samples reacted at room temperature (22 ± 3 °C, discussed above). The results indicate that chlorination of plant material is negligible or absent at high temperatures (spectra not shown). This suggests that the temperatures used in this investigation may be high enough to denature the enzyme, rendering it inactive. NEXAFS spectra obtained from plant needles oven-dried at elevated temperature (~50 °C for 24 h) indicate that the coordination of Cl^- changes from a hydrated state in fresh needles to H-bonded Cl^- in dried needles (Figure 4B,E). The spectral signatures of oven-dried plant needles closely resemble the spectrum of the glycine hydrochloride standard (Figure 4E,F). Air-dried 125-year-old moss samples (*Meteorium tricophorum, Meteorium marriense*) obtained from Princeton University's paleobotanical collection also showed the same spectral signatures.

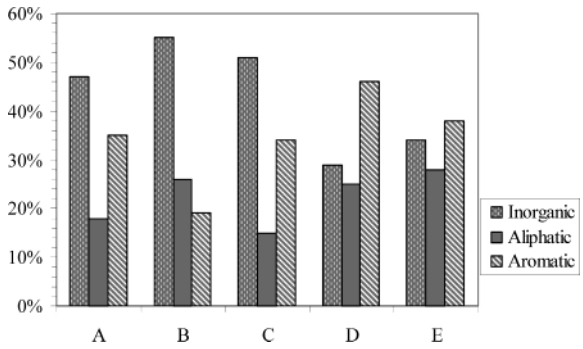

FIGURE 5. Cl Speciation of phenol model system and naturally and artificially weathered redwood needles (from the least-squares fitting of NEXAFS spectra). (A) Phenol + CPO + NaCl + H_2O_2, (B) redwood needle pulp + 50 mM HOCl after 2 d, (C) needle pulp + CPO, (D) needle pulp + CPO + H_2O_2, (E) humified needles on the ground.

This suggests that sample drying at low temperatures can only dehydrate the samples but not chlorinate the organic molecules.

Cl Speciation in Naturally Weathered Plant Material. The NEXAFS spectra of naturally weathered redwood needles show a broad range of Cl speciation, which varies with the intensity of weathering of the plant material (*6*). Weathered redwood needles collected from the O horizon of soil showed a total organochlorine concentration in the range of 50−80%, with aromatic organochlorine in the range of 40−50% (Figure 3). Interestingly, senescent redwood needles collected from the plant (either attached to the tree or detached but hanging on the tree) showed aromatic organochlorine in the range of 0−66% with only traces of aliphatic organochlorine (Figure 3). It is not clear whether differences in weathering reactions or inadequacy of samples are responsible for the observed disparity. However, a strong similarity is noted for the organochlorines in naturally weathered samples and laboratory-reacted model and plant needle samples (Figure 5). All these samples exhibit an absorption maximum at 2821.4 (± 0.1) eV, with organochlorine content ranging from 33% to 80% of the total chlorine. The organochlorine formation in laboratory samples is clearly related to enzyme and oxidant concentration and reaction duration, and these laboratory parameters translate easily into variables affecting bacterial and fungal transformation of plant material in the environment. The diverse distribution of chlorine species arising from various reaction protocols resonates with the high degree of variability witnessed in the organochlorine composition of naturally weathered samples (Figures 2 and 3).

Discussion

The CPO-catalyzed formation of organochlorines from Cl^- in fresh plant material supplies an important piece of the natural organohalogen puzzle. A striking similarity to naturally weathered plant samples is noted among the NEXAFS spectra of a phenol/CPO model system and of fresh plant material reacted with the haloperoxidase enzyme and its probable reaction intermediate HOCl. This suggests that phenolic moieties in plant material are prone to electrophilic attack during the initial stages of chlorination. The nearly identical NEXAFS spectra collected for the phenol/CPO and naturally and artificially weathered plant samples provide additional evidence in support of an electrophilic substitution targeting hydroxyl-rich, aromatic plant matter constituents. Chlorination of these organic substrates likely occurs concurrently with oxidation, either via CPO (alongside its halogenation capabilities, CPO also exhibits a competitive peroxidative function; *24*) or through another route. Previous kinetic investigations have also revealed that chlorination of aromatic groups occurs more rapidly than that of aliphatic components. For example, chlorination of phenol is found to occur rapidly, and its ring opening and chlorination of the resulting small-chain hydrolysis products in the presence of HOCl constitute the overall rate-determining step in the production of chloroform and haloacetic acids (*25*, *26*). Such a process could account for the increase in aliphatic organochlorine that arises in plant material at some point between senescence and humification, a proportion that remains roughly constant as weathering progresses (*6*).

Pulping of plant needles accelerated halogenation reactions in the presence of CPO and of HOCl. This suggests that the aliphatic components dominant in the cuticle of healthy redwood needles make the plant material kinetically lethargic with respect to halogenation. Pulping may expose labile chlorination substrates, such as phenols and other aromatic-rich molecules, to halogenation reactions and likely accelerates CPO-mediated chlorination in plant material by stimulating production of H_2O_2 (*28*). Studies have shown that CPO does not catalyze the chlorination of simple organic substrates in the absence of an oxidant source (*21*, *27*); however, oxidant addition was not required for chlorination when enzyme-containing pulp samples were allowed to react. Interestingly, plants also produce H_2O_2 and other reactive oxygen species specifically to counter fungal and microbial invasions (*29*, *30*). The addition of small amounts of H_2O_2 apparently enhances the halogenation reaction by supplementing plant-based oxidant supplies. The incremental addition of the oxidant in these experiments is consistent with such enzyme-mediated reactions occurring in nature, as lignolytic fungi also use extracellular oxidases to produce H_2O_2 (*31*).

Our studies also indicate that temperature plays a major role in CPO-catalyzed organic molecule halogenation. Prior research has shown a marked decline in CPO activity when the enzyme is heated to 70 °C for even a short time (*13*); therefore, CPO-mediated organochlorine formation is not anticipated at very high temperatures. However, the lack of observed Cl^- conversion in redwood pulp experiments at ∼40 °C suggests that there may be climate-related differences in enzymatic organohalogen production rates (e.g., in boreal versus tropical forest environments). The enzyme activity is also greatest in the pH range of 3−4 (*13*, *21*, *33*), and the highest concentration of organohalogens may be found in acid environments, such as acid soils and the O horizons of all soils where organic matter decomposition buffers the soil pH at low values and supplies organic substrates for halogenation. Recent findings by Oberg (*34*) indicated that organohalogen concentration in soils decreased markedly at pH values above this optimum, which is consistent with the notion that haloperoxidative enzymes contribute significantly to organohalogen production in soils.

It is unclear at present how time allowed for reaction, CPO concentration, and H_2O_2 additions are related and combine to affect the final product composition in laboratory-aged samples. An enzyme exhibiting haloperoxidase-like activity in soil has been detected at bulk concentrations as high as 6.8 u kg^{-1} (*32*), and an isolated soil enzyme has been shown to chlorinate organic substrates (*13*). While CPO concentrations used in this study are much higher than those detected in soil bulk samples, it is not unreasonable to assume that enzyme concentrations in soil microenvironments could exceed previously detected levels. In addition, halogenation of organic substrates observed on the time scales of a few hours to days in the laboratory may take several months because of low CPO concentration in soils and sediments.

An increasing body of research implicates CPO and similar enzymes in a very common and widespread process that may contribute significantly to the global organohalogen burden. It is known that CPO-like enzymes are present in forest soils and exhibit optimum organic chlorination rates

under conditions likely to be found in such soils. Myneni's discovery (*6*) of organochlorines in degrading senescent leaves still attached to the tree, in combination with the present results, suggests that a potent chlorinating agent, most likely an enzyme exhibiting haloperoxidase activity, is present not only in soils but throughout the forest environment. Humic substances in soils and natural waters, a significant portion of which are halogenated (*6*, *35*), very likely derive from direct lignin degradation products such as phenols and quinones (*36*). Since chlorination begins to occur early in the process of plant weathering, it is feasible that Cl^- is incorporated into cell wall lignin molecules, beginning at the onset of senescence and continuing through eventual humification. The proposed CPO-catalyzed chlorination of lignin is consistent with reported studies on microbial lignin degradation (*37*), in which it was shown that addition of reactive chlorine to lignin samples enhanced mineralization by white-rot (lignolytic) fungi. The mechanism of CPO, believed to involve release of the active chlorinating agent into the surrounding medium without requiring that the organic substrate be bound at the active site of the enzyme (*38*), supports the possibility of halogenation of macromolecular structures. Cleavage and chlorination of lignin dimers by CPO in the laboratory also implicates this enzyme in lignolysis as well as the production of chlorinated aromatic macromolecules (*39*). To the extent that not all chlorinated lignins undergo mineralization, the observed net accumulation of natural organohalogens (e.g., chlorinated humics) may be partially explained. Much remains to be discovered regarding the stability of such compounds and their impact on the cycling of chlorine in the environment.

Acknowledgments

This investigation is supported by grants from DOE-BES (Chemical and Geosciences Program) and NSF (Chemical Sciences Program). We are grateful to Big Basin Redwoods State Park Rangers for sampling permission and to the SSRL staff and scientists for assistance with the synchrotron studies. SSRL is administered by Stanford University and supported by the DOE.

Literature Cited

(1) Asplund, G.; Grimvall, A.; Pettersson, C. *Sci. Total Environ*. **1989**, *81/82*, 239−248.
(2) Gron, C. In *Naturally-Produced Organohalogens*; Grimvall, A., De Leer, E. W. B., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1995; pp 49−63.
(3) Oberg, G.; Nordlund, E.; Berg, B. *Can. J. For. Res.* **1996**, *26*, 1040−1048.
(4) Nkusi, G.; Muller, G. In *Naturally-Produced Organohalogens*; Grimvall, A., de Leer, E. W. B., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1995; pp 261−268.
(5) Flodin, C.; Johansson, E.; Boren, H.; Grimvall, A.; Dahlman, O.; Morck, R. *Environ. Sci. Technol.* **1997**, *31*, 2464−2468.
(6) Myneni, S. C. B. *Science* **2002**, *295*, 1039−1041.
(7) Vazquez-Duhalt, R.; Ayala, M.; Marquez-Rocha, F. J. *Phytochemistry* **2001**, *58*, 929−933.
(8) Hodin, F.; Boren, H.; Grimvall, A.; Karlsson, S. *Water Sci. Technol.* **1991**, *24*, 403−410.
(9) Itoh, N.; Izumi, Y.; Yamada, H. *Biochemistry* **1987**, *26*, 282−289.
(10) Wannstedt, C.; Rotella, D.; Siuda, J. F. *Bull. Environ. Contam. Toxicol.* **1990**, *44*, 282−287.
(11) Niedan, V.; Pavasars, I.; Oberg, G. *Chemosphere* **2000**, *41*, 779−785.
(12) Carlsen, L.; Lassen, P. *Org. Geochem*. **1992**, *18*, 477−480.
(13) Asplund, G.; Boren, H.; Carlsson, U.; Grimvall, A. In *Humic Substances in the Aquatic and Terrestrial Environment*; Allard, B., Boren, H., Grimvall, A., Eds.; Springer-Verlag: New York, 1991; pp 475−483.
(14) Hoekstra, E. J.; Lassen, P.; van Leeuwen, J. G. E.; de Leer, E. W. B.; Carlsen, L. In *Naturally-Produced Organohalogens*; Grimvall, A., De Leer, E. W. B., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1995; pp 149−158.
(15) Niedan, V.; Scholer, H. F. *Chemosphere* **1997**, *35*, 1233−1241.
(16) Harper, D. B. *Nature* **1985**, *315*, 55−57.
(17) de Jong, E.; Field, J. A.; Spinnler, H.-E.; Wijnberg, J. B. P. A.; de Bont, J. A. M. *Appl. Environ. Microbiol.* **1994**, *60*, 264−270.
(18) Oberg, G.; Brunberg, H.; Hjelm, O. *Soil Biol. Biochem*. **1997**, *29*, 191−197.
(19) Asplund, G.; Christiansen, J. V.; Grimvall, A. *Soil Biol. Biochem*. **1993**, *25*, 41−46.
(20) Sposito, G. *The Chemistry of Soils*; Oxford University Press: New York, 1989.
(21) Dunford, H. B.; Lambeir, A.-M.; Kashem, M. A.; Pickard, M. *Arch. Biochem. Biophys.* **1987**, *252*, 292−302.
(22) Brown, F. S.; Hager, L. P. *J. Am. Chem. Soc.* **1967**, *89*, 719−720.
(23) Liu, T.-N. E.; M'Timkulu, T.; Geigert, J.; Wolf, B.; Neidleman, S. L.; Silva, D.; Hunter-Cevera, J. C. *Biochem. Biophys. Res. Commun*. **1987**, *142*, 329−333.
(24) Thomas, J. A.; Morris, D. R.; Hager, L. P. *J. Biol. Chem*. **1970**, *245*, 3129−3134.
(25) Gallard, H.; von Gunten, U. *Environ. Sci. Technol.* **2002**, *36*, 884−890.
(26) Fahimi, I. J.; Keppler, F.; Scholer, H. F. *Chemosphere* **2003**, *52*, 513−520.
(27) Walter, B.; Ballschmiter, K. *Fresenius J. Anal. Chem*. **1992**, *342*, 827−833.
(28) Olson, P. D.; Varner, J. E. *Plant J.* **1993**, *4*, 887−892.
(29) Baker, C. J.; Orlandi, E. W. *Annu. Rev. Phytopathol.* **1995**, *33*, 299−321.
(30) Mehdy, M. C. *Plant Physiol.* **1994**, *105*, 467−472.
(31) Aust, S. D. *Environ. Health Perspect.* **1995**, *103*, 59−61.
(32) Laturnus, F.; Mehrtens, G.; Gron, C. *Chemosphere* **1995**, *31*, 3709−3719.
(33) Thomas, J. A.; Morris, D. R.; Hager, L. P. *J. Biol. Chem*. **1970**, *245*, 3134−3142.
(34) Oberg, G. *Water Air Soil Pollut.* **1996**, *89*, 351−361.
(35) Asplund, G.; Grimvall, A. *Environ. Sci. Technol.* **1991**, *25*, 1346−1350.
(36) Stevenson, F. J. *Humus Chemistry*: *Genesis, Composition, Reactions*, 2nd ed.; John Wiley & Sons: New York, 1994.
(37) Johansson, E.; Krantz-Rulcker, C.; Zhang, B. X.; Oberg, G. *Soil Biol. Biochem*. **2000**, *32*, 1029−1032.
(38) Libby, R. D.; Shedd, A. L.; Phipps, A. K.; Beachy, T. M.; Gerstberger, S. M. *J. Biol. Chem*. **1992**, *267*, 1769−1775.
(39) Ortiz-Bermùdez, P.; Srebotnik, E.; Hammel, K. E. *Appl. Environ. Microbiol*. **2003**, *69*, 5015−5018.
(40) Perera, R. C. C.; LaVilla, R. E.; Gibbs, G. V. *J. Chem. Phys.* **1987**, *86*, 4824−4830.
(41) Shadle, S. E.; Hedman, B.; Hodgson, K. O.; Solomon, E. I. *Inorg. Chem*. **1994**, *33*, 4235−4244.
(42) Shadle, S. E.; Hedman, B.; Hodgson, K. O.; Solomon, E. I. *J. Am. Chem. Soc.* **1995**, *117*, 2259−2272.

Received for review July 8, 2003. Revised manuscript received October 1, 2003. Accepted October 8, 2003.

ES0347336