Enhanced removal of natural organic matter via peroxidase-mediated oxidative coupling

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Abstract Oxidative coupling reactions mediated by horseradish peroxidase in solutions containing natural organic matter (NOM) comprised by model fulvic acids were performed in completely mixed batch and flow reactors. The subsequent removal of NOM by ultrafiltration was found to be significantly enhanced for solutions subjected to oxidative coupling. Spectroscopic examinations revealed that the fulvic acid molecules were effectively reconfigured by substantial conversion of aromatic hydroxyl groups into ether bonds via oxidative coupling. These conversions apparently result in cross-linking of NOM moieties to form stable species of greater molecular size, thus rendering them more readily removable by ultrafiltration. The results of this study suggest that catalyzed oxidative coupling reactions in combination with ultrafiltration can be developed into an effective scheme for NOM removal in water treatment operations.

Keywords Catalysis; enzymes; NOM; oxidative coupling; peroxidase; ultrafiltration

Introduction
The natural organic matter (NOM) present in virtually all water sources poses a broad range of problems in water treatment operations and distribution systems (Karanfil et al., 1998; Singer and Reckhow, 1999). These problems relate to the multiple tendencies of NOM species to: i) act as precursors for formation of undesirable by-products during chemical oxidation and disinfection processes; ii) serve as substrates for bacterial growth in filtration and distribution systems; iii) associate with regulated organic chemicals and/or toxic metals and transport them through treatment facilities and water distribution systems; iv) cause taste and odor problems in drinking waters; and, v) deteriorate the efficiencies of activated carbon sorption and membrane filtration processes by causing preloading and fouling problems.

Among the many problems that NOM can cause in drinking water systems, formation of disinfection by-products (DBPs) is perhaps of greatest concern (Singer, 1994; Kitis et al., 2001). Water treatment disinfectants, chlorine in particular, can react with NOMs via a combination of substitution and oxidation mechanisms, yielding fragmented products containing reactive oxidant molecules (Singer and Reckhow, 1999). Common disinfection byproducts include halogenated C1–C3 aliphatics, acids, aldehydes, and ketones, many of which have been shown to be carcinogenic, mutagenic, hepatotoxic, or to cause adverse reproductive and developmental effects.

Current NOM removal methods include enhanced coagulation, granular activated carbon (GAC) sorption, and membrane filtration (Richardson et al., 2002). These methods have proven effective to varying degrees, depending on process conditions and NOM characteristics, but each invariably suffers one or more serious limitations. Coagulation, for example, selectively removes larger NOM macromolecules (i.e. apparent molecular weight >30,000) but is relatively ineffective in removal of smaller ones (Minear and Amy, 1996; AWWA, 1999). Applications of GAC for NOM removal are impaired by difficulties associated with the regeneration of NOM-preloaded carbons. Further, GAC adsorption processes are generally designed to remove specifically targeted organic contaminants, and
NOM adsorption is undesirable in this regard because it can severely decrease GAC performance by preloading active sites prior to their exposure to the contaminants targeted for removal (Karanfil et al., 1996a and b; Weber, 2004). A number of studies have demonstrated that satisfactory NOM removal can be achieved by nanofiltration (NF) and reverse osmosis (RO), but the pores of membranes used in ultrafiltration (UF) and microfiltration (MF) are too large to reject many NOM constituents (Minear and Amy, 1996; Chellam, 2000). The costs associated with NF and RO processes are generally much greater than those of UF and MF because of the higher driving forces and more demanding maintenance schedules required. The cost disadvantages of NF and RO are exacerbated by the fact that most NOM constituents fall in a size range that can readily cause membrane fouling (AWWA, 1999).

There is scientific evidence that NOM molecules may under appropriate conditions be effectively cross-linked by oxidative coupling (Piccolo et al., 2000; Cozzolino and Piccolo, 2002). Oxidative coupling comprises a class of reactions that facilitate the polymerization of molecules having phenolic or anilinic features. These reactions can be catalyzed by a variety of naturally-occurring extracellular enzymes and mineral oxides, and are in fact central to natural humification processes, leading to the formation and growth of soil organic matter from smaller building-block moieties (Bollag, 1992a and b; Huang et al., 2002). Oxidative coupling reactions can lead to significant increases in NOM sizes via covalent linkages, thus rendering them more readily removed in water treatment by processes such as UF/MF or coagulation. The work described here was initiated as a first logical step toward establishing the feasibility of applying catalyzed oxidative coupling reactions in water treatment processes to enhance the removal of NOM.

**Materials and methods**

Suwannee River Fulvic Acid (SRFA) samples were obtained from the International Humic Substances Society (IHSS). Canadian Peat Fulvic Acid (CPFA) was prepared in our laboratories using standard IHSS protocol. The fulvic acids were reconstituted in a 10.0-mmol l⁻¹ phosphate buffer solution (pH = 7.0), filtered through 0.45-µm membranes, and stored in the dark at 4°C prior to use in the experiments. Extracellular horseradish peroxidase (HRP, type-1, RZ = 1.3) was obtained from Sigma Chemical Co., and enzyme activity was measured by the ABTS method (Weber and Huang, 2003; Putter and Becker, 1983). Centrifugal ultrafiltration devices equipped with membranes having nominal molecular weight cutoffs of 3,000 daltons (3 k membrane) and 10,000 daltons (10 k membrane) were obtained from Pall Life Sciences (Ann Arbor, MI). The membranes employed were low protein binding bio-inert types composed of polyethersulfone on a polyethylene substrate. The membranes were conditioned prior to use by soaking in milliQ water for 48 hrs and then passing 3 mL of water through them.

Experiments in completely mixed batch reactors (CMBRs) were performed at room temperature in 13×100-mm glass test tubes. Each reactor contained 6 mL of fulvic acid solution, 2 mmol l⁻¹ of H₂O₂, and varying amounts of HRP. After one hour of incubation, a 3-mL aliquot of the reaction solution was transferred to a centrifugal ultrafiltration device and centrifuged at 10,000 g for 100 min to pass the entire solution through the membrane.

Experiments in semicontinuous flow reactors (SCFRs) were performed in Erlenmeyer flasks. Each reactor contained 100 mL of fulvic acid solution, into which HRP (100 unit/hr) and hydrogen peroxide (0.2 mmole hr⁻¹) were injected continuously at constant rates using KD Scientific syringe pumps. A 3-mL sample was taken from the reactor every hour, equaling the total solution volume being injected into the reactor over the previous 1-hour time interval, thus maintaining the reaction solution volume at 100 mL. The sample was then filtered employing the procedure described for the batch reactor experiments. Blank
tests were performed with either H$_2$O$_2$ or HRP influent absent, but instead with an influent of background 10-mmol l$^{-1}$ phosphate buffer at equivalent flow rate so that volume variations over time were the same as those of the reaction experiments.

The TOC concentrations of NOM solutions were measured using a Shimadzu 9000 TOC analyzer. Ultraviolet (UV) visible spectra were obtained over the wavelength range 200–700 nm with a 1-nm resolution using a Jenway 6405 Ultraviolet/Visible Spectrophotometer and a quartz cuvet having a 1-cm light path. FTIR spectra were obtained using a Thermo-Nicolet Nexus 670-ESP FTIR analyzer. A horizontal attenuated total reflectance (HATR) device containing a ZnSe prism was used as the sample interface. Samples of 100 µL volume were spread on the surface of the ZnSe crystal and blown completely dry by mildly warm air prior to analysis. Additional tests were performed to confirm that water and H$_2$O$_2$ remaining in the test solutions were evaporated completely during sample preparation to prevent potential interferences in the FTIR analyses.

Results and discussion

CMBR investigations

Figure 1 presents data for TOC removal by filtration of NOM solutions subjected to oxidative coupling reactions at different HRP activities through the 3 k and 10 k membranes. The original NOM solutions used for these reactions contained 42.5 mg TOC/L of Suwannee River Fulvic Acid (SRFA). The reactions were performed in CMBRs having an initial H$_2$O$_2$ concentration of 2 mmol l$^{-1}$ and varying activities of HRP. The “TOC removal” data shown in Figure 1 was calculated by comparing TOC concentrations in the NOM solutions after filtration with those of the same solutions before filtration (i.e. 1 – TOC after filtration/TOC before filtration). It thus reflects the filtration removal efficiencies of all organic carbon present in the solution, including the fulvic acids and the enzyme. Additional tests indicate that the 3 k membrane retains the HRP enzyme nearly completely, the average molecular weight of which is reported to be around 20 k. A “fulvic TOC removal” was thus calculated and reported for 3 k membrane in Figure 1 by comparing the TOC remaining in the filtrate with the TOC in the original NOM solution to which no HRP had been added (i.e. 1 – TOC after filtration/TOC before reaction). Assuming the TOC present in the filtrate is attributable solely to fulvic acids, the term “fulvic TOC removal” reflects the removal of fulvic acids by the combined reaction/filtration processes, the values for which are similar to those for overall TOC removal because the contribution of enzyme to TOC is relatively minor.

It is evident in Figure 1 that TOC removals by a 3 k membrane were significantly greater for NOM solutions treated with HRP-mediated reactions than for untreated solutions. No impacts of treatment on TOC removal by a 10 k membrane are evident, however, suggesting that the stable molecular sizes of NOMs produced by oxidative coupling were intermediate between the pore sizes of the 3 k membrane and 10 k membranes.

It is noteworthy that no precipitated product was formed from the oxidative coupling of the fulvic acid NOM, which may relate to the relatively hydrophilic nature of this material. Similar results were observed in earlier experiments on phenol coupling in solutions containing different natural dissolved soil organic matter (Huang and Weber, 2004). While significant amounts of precipitated product were formed by phenol coupling mediated by HRP in solutions containing no dissolved soil organic matter, precipitation was largely reduced in solutions that did, even though phenol conversion was actually enhanced by such organic matter. The oxidative coupling of phenol in NOM-containing solutions thus appears to lead to incorporation of phenol into soluble organic moieties by cross-coupling, resulting in reduction of precipitation.
It is known that HRP can also mediate the dismutation of \( \text{H}_2\text{O}_2 \), yielding products that can bind to the enzyme to form inactive species (Nicell, 1994). This “side effect” is largely suppressed by the presence of phenolic substrates that have high affinities for the enzyme and can thus compete with \( \text{H}_2\text{O}_2 \) for active enzyme sites (Choi et al., 1999). Large steric hindrances are generally associated with large molecules, and the affinities of NOM molecules for HRP may thus not be as high as those of phenol and other phenolic monomers. It is therefore expected that the \( \text{H}_2\text{O}_2 \) dismutation effect may be relatively stronger in NOM reaction systems than in those of other smaller phenolic substrates. Because the \( \text{H}_2\text{O}_2 \)-based side effect is essentially a reaction between HRP and \( \text{H}_2\text{O}_2 \), increases in the concentrations of either would increase the side effect, and thus reduce catalytic efficiency. This may explain the curvilinear trace of the TOC removal data for different enzyme activities observed in Figure 1.

**SCFR investigations**

To overcome the \( \text{H}_2\text{O}_2 \)-based side effect, oxidative coupling reactions of NOM were performed in semi-continuous flow reactors (SCFRs) to which both HRP and \( \text{H}_2\text{O}_2 \) were continuously injected into the reacting NOM solutions. It has been reported that such reactor operations effectively limit the \( \text{H}_2\text{O}_2 \)-based side effect and enhance the overall reaction efficiency (Nicell, 1993). Figure 2 compares 3 k membrane filtration removal efficiencies for SRFA (A) and CPFA (B) solutions treated by oxidative coupling reactions in SCFRs and those of control samples. The reaction samples were prepared SCFRs with continuous injections of HRP and \( \text{H}_2\text{O}_2 \). Control samples were prepared using the same reactor setup, but with only HRP or \( \text{H}_2\text{O}_2 \) being injected. Fulvic TOC removal was calculated as for the CMBR samples, except that dilutions resulting from HRP and \( \text{H}_2\text{O}_2 \) injections were taken into account. It is evident in Figure 2 that the fulvic TOC removal efficiencies for the treated SRFA and CPFA solutions are both significantly greater than those for the control samples, and that they correlate with HRP activity. Greater than 90% removal of fulvic TOC was reached when more than four units/mL of HRP were used, while removals for the control solutions were merely 6–7%.

![Figure 1](https://iwaponline.com/ws/article-pdf/4/4/33/417490/33.pdf)

**Figure 1** TOC removal from SRFA solutions subjected to HRP-mediated coupling reactions in CMBRs at varying enzyme activities. Reaction conditions: initial SRFA concentration = 42.5 mg TOC L\(^{-1}\), \( \text{H}_2\text{O}_2 \) dosage = 2 mmol l\(^{-1}\).
As noted earlier, UV light absorbance has been found to provide a better surrogate measure of DBP formation potential than TOC. It is thus interesting to examine the UV absorbance changes of NOM solutions as affected by the combined reaction/filtration (CRF) process described here. Figure 3 compares the UV absorbances of 3 k-membrane filtrates of the SRFA solutions treated by coupling reactions in SCFRs, with one and eight units/mL of HRP added, to that of the original NOM solution. It can be seen that the UV absorbance of the NOM solution was effectively reduced by the CFR processes. The increases in UV absorbance in the short wavelength range shown in Figure 3 for the reaction samples may be related to the accumulation of H$_2$O$_2$ over the course of the SCFR experiment. In an additional test we confirmed that there was a post-reaction H$_2$O$_2$ level of about 3 mmol l$^{-1}$ remaining in the sample to which 8 units/mL of HRP had been added. Despite the interference imposed by the residual H$_2$O$_2$ on UV analyses, a general trend of reduction can still be seen clearly in Figure 4 for UV-absorbance measured at three specific wavelengths for 3 k membrane filtered samples collected over the course of the SCFR reactions.

**FTIR spectra**

Figure 5 presents FTIR spectra for reaction and control samples of SRFA solutions.

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**Figure 2** Fulvic TOC removal by 3 k membrane filtration of SRFA (A) and CPFA (B) solutions treated in SCFRs with HRP and/or H$_2$O$_2$ injected at the rates of 1 unit ml$^{-1}$ hr$^{-1}$ and 2 mmol hr$^{-1}$ respectively. The TOCs of the original NOM solutions were 47.8 mg L$^{-1}$ for SRFA and 46.3 mg L$^{-1}$ for CPFA.

**Figure 3** UV absorbances of 3 k-membrane filtrates of SRFA solutions treated by oxidative coupling in SCFRs with 1 and 8 units/mL of HRP

**Figure 4** UV absorbances at three specific wavelengths for 3 k-membrane filtrates of SRFA solutions treated by oxidative coupling in SCFRs.
collected from SCFR experiments involving treatment at an HPR dosage of 8 units mL⁻¹. The reaction sample was prepared with continuous injections of both HRP and H₂O₂, while the control sample was made by addition of HRP alone without H₂O₂ and a buffer solution to make up volume. The organic concentrations contained in the reaction and control samples were confirmed by TOC analysis to be nearly the same.

To more closely examine the effects of oxidative coupling on the molecular structures of NOMs, FTIR spectra at four specific wave number regions were quantified. The structural assignments of these four spectrum regions are described in detail in Table 1 (Coates, 2000). The integrated absorbance areas from the FTIR spectra of the reaction and control samples are also compared in Table 1. To account for absorbance variations from sample to sample, the integrated areas listed in Table 1 are normalized by the area of each sample for the aromatic carbon region (ArC, 1,615–1,580 cm⁻¹). The specific absorbance areas thus obtained are compared in Figure 6 for the control and reaction samples. Figure 6 clearly
shows a sharp reduction of aromatic hydroxyl and an increase of aromatic ether responses in the reaction samples. This can be taken as a strong indicator of the occurrence of cross-linking of NOM moieties by conversion of aromatic hydroxyl groups into ether bonds. It is not expected that these oxidative coupling reactions would significantly alter the aliphatic components of the NOM, and the FTIR absorbances specific to aliphatic structures in Figure 6 confirm this.

**Conclusions**
This study shows that oxidative coupling of fulvic acid NOM can be induced in HRP-mediated reaction systems. The reactions are shown to lead to increases in the stable molecular sizes of the NOM, thus enhancing its removal by ultrafiltration. Spectroscopic examinations reveal that substantial conversion of aromatic hydroxyl groups into ether bonds occurs when NOM molecules are subjected to oxidative coupling. These conversions result in cross-linking of the NOM moieties, explaining the mechanism by which they increase in molecular size. The results of the work described here lends strong support to our hypothesis that induced oxidative coupling in combination with ultrafiltration has promising technical potential as a water treatment scheme for NOM removal.

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**References**

### Table 1
Quantitative comparisons of FTIR spectra absorbance areas at four specific wavenumber regions for a control and a reaction sample collected in SCFRs dosed at 8 units mL^{-1} of HRP

<table>
<thead>
<tr>
<th>Region (cm^{-1})</th>
<th>Assignments*</th>
<th>Functionalities</th>
<th>Symbols</th>
<th>Absorbance area</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,640–3,530</td>
<td>Phenolic O-H stretch</td>
<td>Aromatic Hydroxyl</td>
<td>OH</td>
<td>1,184</td>
</tr>
<tr>
<td>2,935–2,915</td>
<td>C-H stretch in -CH2-</td>
<td>Aliphatic</td>
<td>AliC</td>
<td>1,111</td>
</tr>
<tr>
<td>1,615–1,580</td>
<td>C=C-C stretch</td>
<td>Aromatic</td>
<td>ArC</td>
<td>554</td>
</tr>
<tr>
<td>1,270–1,230</td>
<td>Aryl-O stretch</td>
<td>Aromatic ether</td>
<td>ArO</td>
<td>741</td>
</tr>
</tbody>
</table>

* Assignments are based on Coates (2000)


