Comparative toxicity of hydrogen peroxide, hydroxyl radicals, and superoxide anion to \textit{Escherichia coli}

Richard J. Watts, Diana Washington, Jimmy Howsawkeng, Frank J. Loge, Amy L. Teel*

\textit{Department of Civil and Environmental Engineering, Washington State University, Pullman, WA 99164-2910, USA}

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\textbf{Abstract}

The toxicity of hydrogen peroxide, hydroxyl radical, and superoxide radical anion to \textit{Escherichia coli} was investigated as a basis for understanding the effects of hydrogen peroxide when it is injected into the subsurface for in situ bioremediation or in situ chemical oxidation. Hydrogen peroxide toxicity was evaluated by maintaining its steady state concentration at a series of concentrations ranging from 0.7 to 3.0 mM in the presence of \textit{E. coli}. Hydroxyl radical toxicity was studied by conducting parallel reactions of equal steady state concentrations of hydrogen peroxide, but using an iron (III)–nitrilotriacetic acid complex to decompose hydrogen peroxide to hydroxyl radicals. Superoxide was also generated from equal steady state concentrations of hydrogen peroxide, but with the addition of pyrolusite (manganese oxide) to catalyze its decomposition to superoxide radical. Hydrogen peroxide was toxic to \textit{E. coli} at all concentrations investigated. The generation of hydroxyl radicals in hydrogen peroxide solutions showed no increase in toxicity relative to hydrogen peroxide toxicity, indicating minimal additional toxicity of the hydroxyl radicals. Hydrogen peroxide solutions of equal concentrations in which superoxide was generated showed less toxicity relative to hydrogen peroxide systems. The results indicate that toxicity of hydrogen peroxide to microorganisms may be lower when it is injected to subsurface systems containing high manganese oxide contents.

\textbf{Keywords:} Bioremediation; \textit{Escherichia coli}; Fenton’s reagent; Hydrogen peroxide; Hydroxyl radical; Superoxide; In situ chemical oxidation; Toxicity

1. Introduction

Bioremediation has been effective in treating organic contaminants in soils and groundwater through in situ and ex situ processes (Hinchee et al., 1994; Cookson, 1995). Because in situ aerobic bioremediation systems are often oxygen limited, stabilized hydrogen peroxide has been injected into the subsurface as an oxygen source, where it decomposes primarily through reactions with the bacterial enzyme catalase, yielding \( \text{O}_2 \) and \( \text{H}_2\text{O} \) (Spain et al., 1989). Hydrogen peroxide decomposition in soils can also proceed through Fenton-like reactions catalyzed by soluble iron, iron oxides (e.g. FeOOH), or manganese oxides producing transient oxygen species, such as hydroxyl radicals (OH\(^-\)) and superoxide radical anion (\( \text{O}_2^\cdot^- \)) (Watts, 1998). Reactions catalyzed by soluble iron using low concentrations of hydrogen peroxide produce hydroxyl radicals (Walling and Johnson, 1974). The use of iron chelates has recently become popular for oxidizing organic contaminants at neutral pH. Iron–nitrilotriacetic acid (NTA) complexes are an established catalyst for generating hydroxyl radicals at neutral pH (Pignatello and Baehr, 1994). In some subsurface regions manganese oxide nodules may play a significant role in hydrogen peroxide decomposition. The manganese oxide-catalyzed decomposition of hydrogen peroxide generates primarily superoxide (\( \text{O}_2^\cdot^- \)) (Hasan et al., 1999). Superoxide is a weak reductant capable of reducing perhalogenated species,
such as carbon tetrachloride and hexachloroethane (Watts et al., 1999; Teel and Watts, 2002).

Recent results have shown that heterotrophic bacterial metabolism can coexist with hydroxyl radical-mediated oxidations (Howseweng et al., 2001), raising the possibility of using combined abiotic–biotic processes for the treatment of organic contaminants. In such coexisting processes, transient oxygen species would transform the parent compound and aerobic heterotrophic metabolism would transform the degradation products. However, bacterial metabolism is limited under such coexisting conditions because hydrogen peroxide is toxic to microorganisms (Barnes et al., 1996; Buyuksonmez et al., 1998; Freese et al., 1967; Krapp et al., 1997; Vallyathan, 1994; Wolff et al., 1986). The toxicity of hydroxyl radicals generated in vivo has been well documented (Nunoshiba et al., 1999); in addition, the toxicity of hydroxyl radicals generated in the bulk solution has also been documented. For example, Bayliss and Waites (1979) found that an ultraviolet light (UV)/hydrogen peroxide system, which generates hydroxyl radicals, was 2000 times more effective in inactivating Bacillus subtilis than UV irradiation alone. Wolfe et al. (1989) found that ozone/hydrogen peroxide systems were effective in inactivating Escherichia coli and MS2 coliphage. Hydroxyl radicals generated by titanium dioxide photocatalysis were effective in disinfecting water with E. coli (Ireland et al., 1993) phage MS2 (Sjogren and Sierka, 1994), and Clostridium perfringens (Butterfield et al., 1997).

The reactions of superoxide with many biological molecules have been described in detail (Afanas’ev, 1989). Although superoxide toxicity has been measured extensively in vivo (Lynch and Fridovich, 1978; Farr et al., 1986; Benov and Fridovich, 1999), few toxicity studies have been conducted in which superoxide is generated in the bulk phase, such as through the mineral-catalyzed decomposition of hydrogen peroxide that would occur during bioremediation. Therefore, the purpose of this research was to investigate the potential toxicity to E. coli of superoxide generated by a manganese dioxide-catalyzed Fenton-like reaction and to compare superoxide toxicity to that of hydroxyl radicals and hydrogen peroxide.

2. Methods

2.1. Materials

Iron (III) sulfate, NTA, and the components of M9 minimal media (Provence and Curtiss, 1994) were purchased from Sigma-Aldrich. Ascorbic acid, peptone, glucose, noble agar, and nutrient broth were purchased from Fisher Scientific. Pyrolusite (MnO₂) crystals, obtained from D.J. Minerals, were crushed to a fine powder using a 150 ml-capacity Spex shatter box with a hardened steel grinder. The pyrolusite surface area was 5.2 m²/g, which was quantified using a Coulter SA 3100 particle counter. Hydrogen peroxide (50%) was provided gratis by Solvay Interox. E. coli (ATCC No. 35218) was purchased from American Type Culture Collection. Deionized water was purified to 18 MΩ·cm using a Barnstead Nanopure II deionizing system.

2.2. Bacterial culture

Freeze-dried E. coli was inoculated into nutrient broth and spread on nutrient agar plates. After the plates were evaluated for contamination, isolates were collected and grown in nutrient broth. At late log phase the bacteria were centrifuged at 10 000 rpm, rinsed in M9 media 3 times, and resuspended in a dilute peptone solution. The organisms were then stored by deep freezing (Gerhardt et al., 1994).

Prior to each toxicity assay, the bacteria were removed from the freezer and inoculated into 1-l flasks containing 0.5 l of 8 g/l nutrient broth and 1 g/l glucose, which were stirred at 150 rpm for 24 h at 35 °C. The suspended biomass was harvested at late log phase by centrifugation at 10 000 rpm and resuspending in 0.2 l 0.001% peptone solution. The cell concentration after this procedure was 10¹⁰ cfu/ml, which was verified by spread plate counts.

2.3. Solutions and media

A 28 mM glucose medium containing 0.1% M9 media was used to provide carbon for bacterial growth because glucose is unreactive with superoxide (Bielski et al., 1985). Ascorbic acid was used in manganese oxide-catalyzed experiments as a hydroxyl radical scavenger (Buxton et al., 1988). A concentration of 1.4 M ascorbic acid provided a 50:1 molar ratio of ascorbic acid to glucose; the pH was adjusted to 7 using 4 M sodium hydroxide or 2 M sulfuric acid. Serial dilutions were prepared using 0.01% peptone solutions and agar plates were prepared using 8 g/l nutrient broth and 15 g/l noble agar. Iron (III)–nitrilotriacetic acid (Fe–NTA) catalyst stock solutions were prepared by dissolving NTA in deionized water with concentrated sodium hydroxide, adding iron (III) perchlorate in a 1:1 molar ratio, and adjusting the pH to 6 (Pignatello and Baehr, 1994).

2.4. Experimental procedures

All toxicity assays were conducted in a sterile laminar flow hood using sterile 40 ml borosilicate vials capped with PTFE-lined septa. Hydrogen peroxide toxicity experiments contained 2 ml suspended E. coli in 0.01% peptone, 2 ml glucose solution, 2 ml ascorbate solution,
2 ml deionized water, and 2 ml hydrogen peroxide at selected initial concentrations, for a total volume of 10 ml. Hydroxyl radical toxicity experiments contained the same reagents as the hydrogen peroxide toxicity experiments, with the exception that the ascorbate solution was replaced by 2 ml of 5 mM Fe–NTA catalyst. Superoxide toxicity experiments contained the same reagents as the hydrogen peroxide toxicity experiments, with the addition of 0.5 g pyrolusite. Control reactions were conducted in parallel to each experiment, replacing the 2 ml hydrogen peroxide solution with deionized water. As the reactions proceeded, a steady state concentration of hydrogen peroxide (\(\pm 2\%\) of the initial concentration) was maintained throughout the experiments by a constant addition of hydrogen peroxide to the reactors using a syringe pump. Hydrogen peroxide concentrations were determined by visible spectrophotometry after color development with TiSO₄ (Schumb et al., 1955). To verify the effect of superoxide on E. coli toxicity, non-steady state conditions were used; the reactors contained the same reagents as the steady state experiments, with two exceptions: (1) the mass of pyrolusite was varied, and (2) the hydrogen peroxide flow rate was constant.

For all experiments, aliquots were collected periodically to quantify E. coli survival. Samples were serially diluted in peptone solution and three plates were prepared from each dilution using standard spread plate techniques (Gerhardt et al., 1994). The spread plates were incubated at 35 °C for 72 h and the colonies were then counted.

3. Results and discussion

The toxicity of a range of steady state concentrations of hydrogen peroxide to E. coli is shown in Fig. 1. While control cultures grew to 135% of the original cell number over the 2-h assay time, all hydrogen peroxide concentrations had a toxic effect, with toxicity increasing as a function of hydrogen peroxide concentration. Even at the lowest concentration of hydrogen peroxide (0.7 mM), greater than 1-log inactivation of E. coli occurred over the 120-min time of the assay, with 6-log inactivation with 3.0 mM hydrogen peroxide. A range of toxicities for hydrogen peroxide have been reported, with values as low as 0.7 mg/l (20.6 μM) hydrogen peroxide resulting in toxicity to microorganisms in soil and groundwater systems. However, the most common concentration of hydrogen peroxide injected into groundwater systems as an oxygen source for bioremediation has been 100 mg/l (2.94 mM) (Pardieck et al., 1992).

To determine whether hydroxyl radicals affected E. coli survival beyond the effects of hydrogen peroxide, 5 mM Fe–NTA was used to catalyze the decomposition of hydrogen peroxide to hydroxyl radicals in experiments in which steady state hydrogen peroxide concentrations were maintained at the same concentrations used with hydrogen peroxide alone; i.e. the same hydrogen peroxide residual was maintained as in the systems with only hydrogen peroxide, but hydroxyl radicals were also generated by the addition of a Fenton’s catalyst. The results, illustrated in Fig. 2, show similar toxicity to that of hydrogen peroxide alone. Although other AOPs, such as titanium dioxide-mediated photocatalysis and UV/hydrogen peroxide and have been effective in disinfecting water and wastewater (Bayliss and Waites, 1979; Ireland et al., 1993; Sjogren and Sierka, 1994; Butterfield et al., 1997), the hydroxyl radicals produced in the modified Fenton’s systems appear to be less effective. Hydroxyl radicals react with most biological molecules at near diffusion-controlled rates (Buxton et al., 1988). However, because of their high reactivity, the hydroxyl radicals produced in the Fenton’s systems of this research likely reacted only in the bulk phase; i.e. these short-lived species react so rapidly that they had minimal potential to diffuse through the lipid bilayer. By analogy, Sedlak and Andren (1994) found that hydroxyl radicals are not capable of oxidizing sorbed contaminants due to their minimal potential for diffusion to the adsorption sites. The cytotoxicity and genotoxicity of hydroxyl radicals have been well documented in vivo (Nunoshiba et al., 1999); however, hydroxyl radicals in these systems are generated inside the cells by one-electron transfer processes (e.g. the uncoupling of oxidative phosphorylation to generate superoxide, followed by conversion to hydro-
Fig. 2. Inactivation of *E. coli* with steady state addition of hydrogen peroxide and 5 mM Fe–NTA (reaction contents: 2 ml suspended *E. coli* in 0.01% peptone, 2 ml glucose solution, 2 ml ascorbate solution, 2 ml 25 mM Fe–NTA, and 2 ml hydrogen peroxide at varying concentrations).

Fig. 3. Inactivation of *E. coli* with steady state addition of hydrogen peroxide and 0.5 g pyrolusite (reaction contents: 2 ml suspended *E. coli* in 0.01% peptone, 2 ml glucose solution, 2 ml ascorbate solution, 2 ml water, 0.5 g pyrolusite, and 2 ml hydrogen peroxide at varying concentrations).

gen peroxide by superoxide dismutase and then Fenton’s reactions to generate hydroxyl radicals). Such intracellular generation of hydroxyl radicals likely produces quite different effects compared to hydroxyl radicals generated by Fenton’s reactions outside the cells.

Superoxide is a weak reductant and nucleophile and has minimal reactivity with many biological molecules (Bielski et al., 1985). To investigate the toxicity of superoxide to *E. coli*, 0.5 g of pyrolusite was used to catalyze the decomposition of hydrogen peroxide to superoxide in experiments with steady state hydrogen peroxide concentrations maintained at the same concentrations used with hydrogen peroxide alone. The results (Fig. 3) show lower toxicity for superoxide and hydrol...
Fig. 4. Comparison of hydroxyl radical and superoxide toxicity, with hydrogen peroxide toxicity subtracted from the data. Steady state concentrations of hydrogen peroxide: (a) 3.0 mM; (b) 2.2 mM; (c) 1.5 mM; (d) 0.7 mM.
gen peroxide together than for hydrogen peroxide alone (Fig. 1) or for hydroxyl radicals and hydrogen peroxide (Fig. 2), particularly at the lower hydrogen peroxide concentration of 0.7 mM. At 0.7 mM hydrogen peroxide alone only 0.2-log E. coli inactivation occurred with the addition of pyrolusite compared to over 1-log inactivation without the addition of the manganese dioxide. At higher hydrogen peroxide concentrations the addition of pyrolusite also resulted in lower toxicity than hydrogen peroxide alone (4-log inactivation vs. 6-log inactivation for hydrogen peroxide alone). The data of Fig. 3 relative to Fig. 1 denote significantly less toxicity of combined hydrogen peroxide and superoxide compared to hydrogen peroxide alone. Superoxide can dismutate to hydrogen peroxide; however, a potential mechanism for superoxide detoxification is the presence of Mn (II) complexes, which function as superoxide scavengers (Archibald and Fridovich, 1982).

To further evaluate the toxicity of superoxide relative to hydroxyl radicals, the background toxicity of hydrogen peroxide was subtracted from the data of Figs. 2 and 3. The relative percentage increase or decrease in survival of E. coli exposed to hydroxyl radicals and superoxide compared to hydrogen peroxide alone is shown in Fig. 4. At the higher hydrogen peroxide concentrations (2.2 and 3.0 mM), hydroxyl radical and superoxide exposures were characterized by toxicities similar to hydrogen peroxide alone for the first 20 min. At 1.5 mM hydrogen peroxide, both hydroxyl radicals and superoxide showed higher survival than hydrogen peroxide for the first 40 min, but were similar by 120 min. At the lowest hydrogen peroxide concentration, 0.7 mM, superoxide was significantly less toxic than hydroxyl radical; by 120 min, the systems exposed to superoxide had 40% greater survival than hydrogen peroxide alone, while survival in hydroxyl radical systems was indistinguishable from hydrogen peroxide alone. These results indicate that superoxide generated by manganese oxide-catalyzed decomposition of 0.7 mM hydrogen peroxide is significantly less toxic than either hydrogen peroxide alone or hydroxyl radicals generated by Fe–NTA-catalyzed hydrogen peroxide decomposition.

To further differentiate the toxicity of superoxide and hydrogen peroxide, the concentration of the pyrolusite catalyst was varied while the injection rate of hydrogen peroxide was held constant. The mass of pyrolusite was varied from 0 to 2 g, while the initial hydrogen peroxide concentration of 0.7 mM was augmented continuously by 0.3 M hydrogen peroxide added at a 1.5 ml/h flow rate. As shown in Fig. 5, the addition of pyrolusite decreased the toxicity of the system. Masses of 0.5, 1.0, and 2.0 g pyrolusite resulted in no more than 1-log inactivation of E. coli over 120 min, compared to approximately 6-log inactivation for hydrogen peroxide alone. These results confirm that superoxide is less toxic than both hydrogen peroxide alone and hydroxyl radicals generated in an iron-catalyzed Fenton-like reaction, and even appears to serve as a detoxifying agent for E. coli exposed to hydrogen peroxide.

Hydrogen peroxide was once used extensively as a source of oxygen for in situ bioremediation. The use of hydrogen peroxide has decreased as a result of the findings of excess hydrogen peroxide decomposition due to high catalase activity in biofilms surrounding injection wells (Spain et al., 1989). However, recent results suggest that the use of hydrogen peroxide as an oxygen source for in situ bioremediation or for direct in situ chemical oxidation may have previously unforeseen advantages. For example, Howsawenk et al., 2001 found that oxidation by hydroxyl radicals can occur in the presence of even high biomass levels. The results of the research reported here provide another mechanism that may benefit abiotic–biotic degradations in hydrogen peroxide-based treatment systems. Since manganese oxides are ubiquitous in the subsurface, they may catalyze hydrogen peroxide decomposition to superoxide, which is less toxic to microorganisms than hydrogen peroxide. Superoxide may also contribute to in situ remediation in other ways; it has been implicated in enhanced contaminant desorption, destruction of dense nonaqueous phase liquids, and the reduction of highly halogenated contaminants, such as carbon tetrachloride (Siegrist and Watts, 2001). Therefore, although both the abiotic and biotic processes occurring in sub-surfaces exposed to hydrogen peroxide are complex, the
results recently obtained strongly suggest that microorganisms can coexist with transient oxygen species.

4. Conclusion

The relative toxicity of hydrogen peroxide, hydroxyl radical, and superoxide radical anion to *E. coli* was evaluated under steady state hydrogen peroxide conditions. Hydrogen peroxide was maintained at steady state concentrations of 0.7, 1.5, 2.2 and 3.0 mM. Hydroxyl radicals were produced through the catalytic decomposition of hydrogen peroxide using an iron (III)–NTA catalyst. Superoxide was generated by the catalytic decomposition of hydrogen peroxide using a pyrolusite catalyst (Hasan et al., 1999). Reactions with hydrogen peroxide alone and with hydroxyl radicals and hydrogen peroxide together exhibited equal toxicity to *E. coli*, indicating that hydroxyl radicals provide no measurable increase in toxicity relative to hydrogen peroxide alone. However, reactions that generate superoxide (while maintaining equal residuals of hydrogen peroxide) were significantly less toxic to *E. coli* than solutions containing hydrogen peroxide alone. The results of this research suggest that when hydrogen peroxide is injected into the subsurface as an oxygen source for bioremediation or as a chemical oxidant, manganese oxide nodules can lower its toxicity to bacteria, providing mechanisms for both abiotic and biotic degradation processes.

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References


Richard J. Watts is a professor in the Department of Civil and Environmental Engineering at Washington State University. His primary area of research is chemical oxidations applied to soil and groundwater systems. He is also author of the text Hazardous Wastes: Sources, Pathways, Receptors published by John Wiley & Sons.

At the time of this research, Diana Washington was a masters student in the Department of Civil and Environmental Engineering at Washington State University. She is currently pursuing a doctoral degree in the same department.

At the time of this research, Jimmy Howsawkeng was a doctoral student in the Department of Civil and Environmental Engineering at Washington State University. He is currently employed by Brown and Caldwell in Nashville, TN.

Frank J. Loge is an assistant professor in the Department of Civil and Environmental Engineering at Washington State University. He received a Ph.D. from the University of California, Davis. His research focuses on chemical oxidants and disinfectants for water quality improvement.

Amy Teel is a Research Scientist at Washington State University. She received her Ph.D. in Molecular, Cell and Developmental Biology from the University of Minnesota in 1996. Since then she has worked with Fenton’s reagent for the treatment of hazardous wastes, including several projects involving combined abiotic–biotic processes.