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Microbial adaptation to hydrogen peroxide and biodegradation of aromatic hydrocarbons

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This research investigated microbial responses to bioremediation with hydrogen peroxide (H_2O_2) as a supplemental oxygen source. Columns containing aquifer material from Traverse City, MI, USA, were continuously supplied with benzene, toluene, ethylbenzene, *o*-xylene and *m*-xylene (BTEX) and H_2O_2 in increasing concentration. The microbial responses studied were changes in microbial numbers, community structure, degradative ability, and activity of catalase and superoxide dismutase (SOD). Both adaptation to H_2O_2 and stress-related consequences were observed. Adaptation to H_2O_2 was demonstrated by increased catalase and SOD activity during the course of the experiment. The microbial community in the untreated aquifer material used in the columns consisted primarily of *Corynebacterium* sp and *Pseudomonas fluorescens*. Following amendment with 500 mg L^{-1} H_2O_2, the column inlet was dominated by *P. fluorescens* with few *Corynebacterium* sp present; *Xanthomonas maltophilia* dominated the middle and outlet sections. Dimethyl phenols detected in the effluent of two of the biologically active columns were probably metabolic products. The ratio of oxygen to BTEX mass consumed was approximately 0.3 before H_2O_2 addition, 0.7 following 10 mg L^{-1} H_2O_2 supplementation, and 2.6 over the course of the experiment. Abiotic decomposition H_2O_2 was observed in a sterile column and impeded flow at a feed concentration of 500 mg L^{-1} H_2O_2. Increasing the BTEX concentration supplied to the biologically active columns eliminated flow disruptions by satisfying the carbon and energy demand of the oxygen evolved by increasing catalase activity.

Keywords: catalase; hydrogen peroxide; superoxide dismutase; adaptation; biodegradation; BTEX

Introduction

In situ bioremediation (ISB) treats contamination where it is found in the subsurface, minimizing cross-media contamination. The technology, as patented by RL Raymond [45,46], supplies nutrients and an oxygen source through injection wells or an infiltration gallery to enhance biodegradation. The treated ground water may be recovered by production wells for recirculation to the treatment zone, if allowed by local regulations, or disposed via the sanitary sewer or other water conduits. Ideally, indigenous microorganisms degrade contaminants dissolved in the ground water and present on aquifer material, however ISB is most effective for the biodegradation of dissolved contaminants.

Optimizing bioremediation through the addition of nutrients and an electron acceptor, which is usually oxygen, generally increases the rate of biodegradation, reduces the risk of exposure to the chemical, and saves time and money. For aerobic bioremediation of hydrocarbons, estimates of the required oxygen mass range from the conservative 3 : 1 ratio, which represents maximum O_2 utilization for complete degradation to CO_2, (as used in the BIOPLUME II model [48] and in bioventing [33]) to the ratio of 1.08–1.7 oxygen : hydrocarbon when the simultaneous production of cell mass is considered [12]. Methods for increasing the dissolved oxygen (DO) concentration in ground water include sparging with air, addition of pure oxygen, and addition of hydrogen peroxide (H_2O_2) or solid metal peroxides. Because the solubility of oxygen is low and the quantity of contaminant is often great, the oxygen supplied by air sparging, and even by pure oxygen equilibration, can be quickly depleted by microorganisms. An alternative method for increasing the DO concentration of an aquifer is the addition of H_2O_2 (see [10] and [44] for reviews). Many laboratory and field experiments have been inconclusive, in part because degradation of complex hydrocarbon mixtures was measured in heterogeneous, contaminated subsurface material. Also, the focus of laboratory and field studies has been largely phenomenological, emphasizing the result rather than the mechanisms controlling the technology.

Because H_2O_2 decomposes to oxygen and water and is soluble in water, ostensibly unlimited amounts of oxygen are available for biodegradation. However, H_2O_2 is subject to decomposition by the action of light, metals, and enzymes; it is somewhat more reactive than oxygen because it has two pairs of electrons in antibonding orbitals compared to oxygen's one pair and also does not have the spin restriction of oxygen. Despite the apparent contradiction of using a potential toxicant to promote biological degradation, subsurface bioremediation supplemented with H_2O_2 has proceeded on the premise that H_2O_2 toxicity would not impair the biodegradation process and its abiotic decomposition could be minimized by pretreatment of an aquifer with metal-complexing agents.

Hydrogen peroxide is not a xenobiotic chemical; it can be formed *in vivo* through a number of pathways. For example, *Streptococcus faecalis* oxidizes glycerol 3-phosphate to dihydroxyacetone phosphate with the concomitant release of H_2O_2 [15]; the cyanobacterium *Anacystis nidulans* generates H_2O_2 during photoautotrophic growth [50].

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Hydrogen peroxide is also the result of the dismutation reaction. Any superoxide (O_2^-) generated enzymatically at physiological pH will dismute to form H_2O_2 [30]. The cellular concentration of superoxide ranges from 10^{-12} M to 10^{-11} M; the concentration of H_2O_2 ranges from 10^{-9} M to 10^{-7} M.

Many microorganisms have developed enzymes to defend against H_2O_2 and mechanisms to repair damage. Aerobic and oxygen-tolerant bacteria possess the enzymes catalase, peroxidase, and superoxide dismutase (SOD) that decompose H_2O_2 and superoxide. The toxicity of H_2O_2 can be attributed primarily to the free radicals, especially the hydroxyl ($OH^\bullet$) radical, formed by its decomposition [30]. The ensuing damage to DNA, which might include single-strand breaks [3], apurinic-apyrimidinic sites [17], and altered bases [18], can be repaired by the appropriate DNA repair system. If the initial DNA damage is reparable, then increased levels of catalase can remove exogenous H_2O_2. Exogenous H_2O_2 induces catalase in *Salmonella typhimurium* [20,62], *Escherichia coli* B [47], *E. coli* K12 [63], a catalase isozyme in *Bacillus subtilis* [40], and the HPI catalase isozyme in *E. coli* [41]. In studies of catalase induction, both SOD and catalase work cooperatively in protecting against oxygen toxicity. Catalase and superoxide dismutase are a mutally protective set of enzymes because each is inhibited by the other's substrate [34,37,54]. To withstand a continuous input of H_2O_2, subsurface microorganisms must possess these enzymes and may synthesize them following exposure.

We simulated a field study of H_2O_2-supplemented ISB [61] by adding H_2O_2 in increasing concentration [46] and benzene, toluene, ethylbenzene, *m*-xylene, and *o*-xylene (BTEX) to columns containing aquifer material from Traverse City, MI, USA. The step-wise increases in H_2O_2 concentration in field applications and in this experiment were designed to foster microbial adaptation. Our research examined the effect of H_2O_2 addition on a subsurface microbial community. Increases in catalase and superoxide dismutase specific activity were studied as indicators of adaptation to H_2O_2. The signs of stress that we investigated were bacterial numbers and community structure. One unequivocal consequence of H_2O_2 toxicity would be a decline in the population size; a less conspicuous effect might be a change in the relative abundance of the bacterial species present while the total number of microorganisms remained constant. A sterile column served as a control and provided evidence of the abiotic reaction of H_2O_2 with the aquifer material, leading to oxygen gas formation and flow disruption. In this report, we present our findings on: the increase in catalase following exposure to H_2O_2, change in community structure and numbers of viable bacteria, the quantities of BTEX, DO, and H_2O_2 recovered after passage through columns of biologically active and sterile aquifer material, the differential biodegradation of BTEX components, detection of metabolic intermediates, and the observed oxygen : BTEX mass utilization ratio.

Materials and methods

Experimental apparatus

A slightly contaminated subsurface core of aquifer material from Traverse City, Core I17, was used in the experiment; core and aseptic sample collection procedures were discussed previously [61]. The subsurface at Traverse City consists of glacial deposits; the shallow sand and gravel aquifer is underlain by clay. Column size and system design considered flow parameters in the unconfined aquifer at Traverse City, MI, subsurface sample availability, and analytical requirements (Figure 1). Water jackets were maintained at 12°C, the *in situ* ground water temperature at Traverse City; a syringe pump (infusion/withdrawal, Harvard Apparatus, South Natick, MA, USA) supplied the feed solution at a constant flow rate of 2.4 ml h^{-1} (one pore volume was 9 ml). The porosity, n, of the water-saturated columns was 0.32 and seepage velocity, V, was 1.18×10^{-3} cm s^{-1} (field values: n = 0.25–0.40, V = $1.06 - 2.12 \times 10^{-3}$ cm s^{-1}, [59]). The use of stainless steel tubing and fittings in the apparatus (0.3175 cm o.d., nuts, ferrules, and connectors, Houston Center Valve and Fitting, Houston, TX, USA) and glass columns (16.5 cm long, 1.5 cm i.d., Spectrum, Houston, TX, USA) and dosing syringes (250 ml, Popper, New Hyde Park, NY, USA) minimized loss of BTEX by adsorption or volatilization. The column end fittings were 316 stainless steel and joined to the glass column with a Viton O-ring; a 10-μm mesh size stainless steel screen (Supelco, Inc, Bellefonte, PA, USA) placed between each end fitting and the column prevented movement of the soil grains but allowed elution of bacteria. The interaction of the column apparatus with H_2O_2 and hydrocarbon was assessed in separate experiments. Loss of neither H_2O_2 nor hydrocarbon was detected. Aquifer material was added to the sterilized apparatus in a sterile, laminar flow chamber to minimize contamination by foreign microorganisms. For the sterile column, 100 g of aquifer material was autoclaved for 30 min on three consecutive days prior to installation.

Column feed solution

The column feed solution contained per liter: KH_2PO_4, O.80 g; Na_2HPO_4, 0.53 g; NH_4Cl, 1.3 g; Na_2CO_3, 0.13 g; $CaCl_2 \cdot H_2O$, 0.013 g; $MgSO_4 \cdot 7H_2O$, 0.27 g; $MnSO_4 \cdot H_2O$, 0.027 g; and $FeSO_4 \cdot 7H_2O$, 0.0067 g (pH 7.0), as a factorial experiment with our water source indicating that these nutrients were necessary for optimum cell growth. The feed solution for the sterile control also contained 0.01 M for-

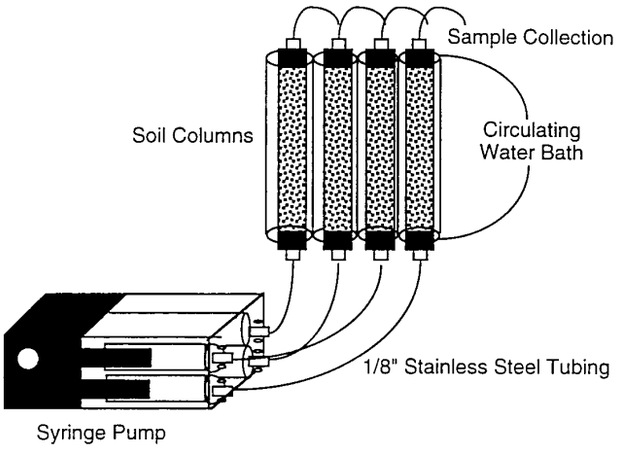

Figure 1 Apparatus used in column experiments.

maldehyde to prevent microbial growth. The dosing solution was prepared to a DO concentration of air saturation, dosed with the appropriate amount of a stock H_2O_2 solution (1 M H_2O_2), transferred to amber bottles, and sealed with a septum cap; no headspace remained. Then, the solution was inoculated with neat benzene, toluene, ethyl benzene, *m*-xylene, and *o*-xylene. The H_2O_2 and BTEX dosing schedule is shown in Table 1. The solutions were stirred at room temperature (22°C) for at least 8 h to allow dissolution of the hydrocarbon contaminants. A syringe containing 250 ml of the solution supplied one of the four columns.

Column material sampling

When sacrificing the columns, the subsurface material was separated based on location in the column: inlet, middle, and outlet. Approximately 12 g was removed from the outlet section (the 'top' of the column, given that flow was in the up direction), homogenized, and then analyzed. The quantity of 12 g allowed for duplicate soil washing/enzyme assays (5 g each), one determination of microbial numbers (1 g), and duplicate soil dry weight measurements (<0.5 g each). Next, another 12 g was removed from the inlet section and distributed for the same analyses as the outlet section. The larger quantity of soil in the middle section permitted duplicate BTEX extractions. One column was sacrificed after each key increase in H_2O_2 concentration. The first live column, Column C, was sacrificed after treatment with 0.1 mg L^{-1} H_2O_2, followed by Column B, which was sampled after amendment with 10 mg L^{-1} H_2O_2. After the addition of 500 mg L^{-1} H_2O_2, Column A, live, and Column S, the sterile control, were sacrificed. Bacterial species were also isolated, identified, and quantified in Column A and then compared with those quantified and identified in untreated core material.

Column material washing protocol, protein and enzyme assays

Five grams of column material and 5 ml of washing solution (0.1% polyvinyl-pyrrolidone, 0.05 M sodium pyrophosphate, pH 7.0) were mixed at 200 rpm for 10 min at 4°C (Psychrotherm, New Brunswick Scientific Co, New Brunswick, NJ, USA); the suspension, which included cells, was removed by pipette. The washing process was repeated for a total of six washes. The cell suspensions were pooled and centrifuged at $10\,000 \times g$ for 10 min to collect the cells. After washing the cell pellet three times in 5 ml of PO_4 buffer (0.05 M PO_4, 0.1 mM EDTA, 0.1 M NaCl, pH 7.0), it was suspended in 5 ml of a solution (0.75 M sucrose, 0.05 M Tris, 5 mM EDTA, pH 7.8) containing 1 mg ml^{-1} lysozyme and incubated for 2 h. The cells and spheroplasts were centrifuged at $10\,000 \times g$ and resuspended in 1 ml of PO_4 buffer for sonication. The cells were sonicated (Heat Systems Ultrasonics, Model W185D, Plainview, NY, USA) for 10 min in 45-s bursts at output 6 using a cup horn attachment; the contents of the centrifuge tube were maintained below 4°C. Cell debris was removed by centrifugation at $5000 \times g$; the supernatant fluid was stored at 4°C prior to protein and enzyme assays.

The protein concentration of the crude extracts was determined by the Bradford method [8], using a prepared reagent (Bio-Rad, Richmond, CA, USA) and bovine serum albumin for the generation of standard curves. Catalase activity was assayed according to a modified method [14], in which the rate of disappearance of a 0.01 M H_2O_2 solution (0.05 M PO_4 buffer, 0.1 mM EDTA, pH 7.0, 25°C) is followed at 240 nm. The extinction coefficient for H_2O_2 is 43.6 M^{-1} cm^{-1}. One unit of enzyme activity is the amount of catalase that degrades 1 μmol of H_2O_2 per min.

An indirect method was used for the measurement of superoxide dismutase activity. The oxidation of xanthine by the enzyme xanthine oxidase produces urate and superoxide. The superoxide then reacts with oxidized Fe(III) cytochrome *c* to yield reduced Fe(II) cytochrome *c* [22]. One unit of superoxide dismutase is the amount that inhibits the rate of Fe(III) cytochrome *c* reduction by 50%. Ordinarily, the crude protein extract is added by trial and error until the amount that gives a rate of 0.0125 min^{-1} is found. Because there was only a limited amount of crude protein extract for the SOD assay (approximately 250 μl of a 0.1 mg ml^{-1} protein solution), the 50% inhibition method could not be used. Sawada and Yamazaki [51] developed a linear relationship that can be used with any inhibition level. Their equation follows:

$$\frac{v}{v_c} = 1 + \frac{k_{SOD}\,[SOD]}{k_c[\text{cyt } c]}$$

$[\text{cyt } c] = 1 \times 10^{-5}$ M, assay condition

Solving the equation leads to an SOD concentration of 2.5×10^{-9} M = 1 unit. The rates observed with the assay were used to determine the SOD concentration of the crude protein extracts in molarity, and then converted to units, according to the following relationship:

$$\frac{0.025}{\text{rate in assay}} = 4.1 \times 10^8\,[SOD];$$

$$[SOD],\ \text{M}, \times \frac{1\ \text{unit}}{2.5 \times 10^{-9}\ \text{M}} = \text{units of SOD}$$

The suitability of this method for the crude protein extracts

Table 1 Treatment schedule for the addition of H_2O_2 and BTEX to the columns

Week	Dose (mg L^{-1}) of:					
	H_2O_2	B	T	E	*m*-X	*o*-X
1	0.0	0.5	0.5	0.5	0.5	0.5
2	0.1	0.5	0.5	0.5	0.5	0.5
3	0.0	0.5	0.5	0.5	0.5	0.5
4	0.1	0.5	0.5	0.5	0.5	0.5
5	1.0	0.5	0.5	0.5	0.5	0.5
6	10	0.5	0.5	0.5	0.5	0.5
7	50	1.5	1.2	0.5	0.5	0.5
7.5	75	1.8	1.8	0.5	0.5	0.5
8	100	3.0	2.3	1.2	1.2	1.2
9	100	11	11	6.0	6.0	6.0
10	250	11–22	11–22	6.0	6.0	6.0
11	500	23–50	23–35	20	20	20

B = benzene, T = toluene, E = ethyl benzene, *m*-X = *m*-xylene, *o*-X = *o*-xylene.

from the column material was confirmed by using three aliquots of one sample that existed in ample supply.

Peroxidase activity was assayed by the rate of formation of the colored product (A_{460}) that results from the oxidation of *o*-dianisidine (*o*-dianisidine dihydrochloride, Sigma) by H_2O_2. The buffer composition was 0.01 M PO_4, 0.1 mM EDTA, pH 6.5, 25°C; the reactant composition was 5 mM H_2O_2 and 0.2 mg ml^{-1} *o*-dianisidine. The extinction coefficient of oxidized *o*-dianisidine is 11 300 M^{-1} cm^{-1}.

Enumeration of microorganisms: microbial community diversity

Column effluent samples were diluted serially in a mineral salts medium that contained per liter: KH_2PO_4, 0.80 g; Na_2HPO_4, 0.53 g; NH_4Cl, 1.3 g; Na_2CO_3, 0.13 g; $CaCl_2 \cdot H_2O$, 0.013 g; $MgSO_4 \cdot 7H_2O$, 0.27 g; $MnSO_4 \cdot H_2O$, 0.027 g; $FeSO_4 \cdot 7H_2O$, 0.0067 g (pH 7.0). Bacteria were released from the column material by shaking 1 g with 9.5 ml of sterile Traverse City, MI ground water at 200 rpm, 4°C, for 10 min. The subsurface material was diluted serially in mineral salts medium.

Even though several methods exist for the enumeration of microorganisms (see [23] for a review), viable bacteria were enumerated in this experiment. Viable counts were determined by the spread plate technique, using 0.1-ml aliquots of serial dilutions of the ground water and subsurface samples. Heterotrophs were recovered on nutrient agar (NA) and diluted nutrient agar (50% dilution, only data for NA viable counts are reported). Higher numbers of heterotrophs might have been detected with a less rich medium than nutrient agar. The number of hydrocarbon-degrading (HD) or -tolerant organisms was determined with 1.5% noble agar amended with mineral salts and incubated under a benzene, toluene, and aviation gasoline (Exxon Refinery, Baytown, TX, USA) atmosphere. All media were purchased from Difco (Detroit, MI, USA). The NA plates were incubated for 1 week at 22°C; the HD plates were incubated for 4 weeks at 22°C.

The numbers of the different morphotypes in the inlet, middle, and outlet sections of Column A (the column that received the highest H_2O_2 dose) were recorded from the enumerated nutrient agar plates. The numbers of the same morphotypes were determined similarly for the untreated material, Core I17. Several colonies of each type from Column A and the untreated material were isolated and streak-plated four times on nutrient agar to ensure a pure culture. The isolates were identified to the genus level by Microbiology Specialists, Houston, TX, USA.

BTEX analysis

Samples from the syringes were collected before their attachment to the columns and at the time of detachment to ensure that the feed solution concentration had not changed and to serve as a duplicate measurement. Column effluent samples were collected over a period of 8–12 h. Both syringe and column effluent samples were collected and concentrated on Sep-Pak C-18 cartridges (Waters Chromatography Division, Millipore Corp, Milford, MA, USA), which were extracted with dichloromethane (HPLC grade, Aldrich, Milwaukee, WI, USA). The extracts were analyzed by gas chromatography with flame ionization detection (GC/FID), (Hewlett-Packard GC Model 5890, Model 2298A integrator, HP-1 column), detection limit = 0.002 mg L^{-1}. The temperature program ran from 30°C to 120°C, with a 1-min initial hold, a 5°/min ramp, and a 1-min final hold; the injector temperature was 225°C and the detector temperature was 300°C. BTEX was quantified by the internal standard method, using 1,2,4-trimethyl benzene (pseudocumene) as the internal standard. The column material was extracted according to a modification of the method of Vandegrift [60]. The extraction efficiency was ascertained by the recovery of 4-methyl isopropyl benzene (*p*-cymene) added as a spike to the soil.

Some of the effluent extracts and all of the middle sections of the column material were analyzed qualitatively by mass spectrometry (Hewlett-Packard GC and Mass Spectrometer, HP-1 column) to confirm the presence of benzene, toluene, ethylbenzene, *o*-xylene, and *m*-xylene and to identify metabolic intermediates. The BTEX compound identifications on the mass spectrometer correlated with the relative retention times on the GC-FID with the HP-1 megabore column. The identity of the isomers of dimethyl- and ethylphenolic intermediates was confirmed by comparison with the retention times and fragment patterns of standards prepared from neat compounds (phenols kit, Alltech Associates, Waukegan, IL, USA).

DO analysis

Dissolved oxygen was measured polarographically with a Clark type electrode (YSI Model 57 Oxygen Meter, Yellow Springs, OH, USA) in a specially fabricated flow-through cell. The meter was calibrated daily with air-saturated samples; the volume and time necessary to achieve air saturation were determined with the Winkler titration [5].

H_2O_2 analysis

Hydrogen peroxide was quantified by the titanium sulfate method [56]; Richard L Raymond Sr of Du Pont Environmental Services provided the $TiSO_4$ reagent. One milliliter of acidified $TiSO_4$ was added to a 10-ml sample; the absorbance at 405 nm was measured with a Gilford Response Spectrophotometer. The concentration in samples was determined by comparison with a calibration curve generated by a linear regression program on a Gilford Response Spectrophotometer (Ciba-Corning Diagnostics, Oberlin, OH, USA). The assay was used for H_2O_2 concentrations ranging from 10 to 500 mg L^{-1}.

Oxygen to BTEX ratio

The ratio of oxygen to BTEX mass used was determined at each feed solution change (a two- to three-day period) and reported in a cumulative manner for specific time intervals. The first interval was from the beginning of BTEX addition through the twelfth day, when the first of the columns (Column C) was sacrificed; the second time interval extended through the addition of 1 mg L^{-1} H_2O_2; the third time interval extended through the addition of 10 mg L^{-1} H_2O_2; finally, O : BTEX was determined for the length of the experiment. Effluent concentrations of each BTEX component were subtracted from influent concentrations to determine the mass of BTEX used. The oxygen mass used was attributed to two input sources, the DO of the feed

solution and the input H_2O_2 concentration. The effluent concentrations of DO and H_2O_2 were subtracted from the influent concentrations, with a factor of 0.47 applied to the H_2O_2 concentration. Because no H_2O_2 appeared in the effluent of the live columns, it was all considered to be used biologically. The assumption of complete biological utilization of oxygen from H_2O_2 may lead to a false high ratio of O : BTEX.

Results

Enzyme activities

Catalase (specific activity, Units mg^{-1} protein) in the inlet and middle sections of the first column sacrificed, Column C, which received 0.1 mg L^{-1} (2.94 μM) H_2O_2 for 5 days, was 20.7 U mg^{-1} and 15.6 U mg^{-1}, respectively. These levels were greater than in the outlet section, 7.3 U mg^{-1} (Table 2). Catalase was increased in the inlet and middle sections of Column C, whereas catalase activity in the outlet section was only slightly higher than that in the untreated material from Core I17, 6.2 U mg^{-1} (data not shown). In Column B, which received 10 mg L^{-1} (294 μM) H_2O_2, the inlet, middle, and outlet sections had specific activities of 14.9, 10.7, and 11.0 U mg^{-1}, respectively. Catalase specific activity in Column B was greater than in the untreated core material, but not as high as that of the inlet and middle sections of Column C. Column A ran for the length of the experiment and at the conclusion, received 500 mg L^{-1} H_2O_2 (14.7 mM) for over 1 week. Catalase-specific activity was elevated in all sections of the column. The catalase in the inlet reached an average of 583 U mg^{-1}; in the mid-section, catalase was 97.5 U mg^{-1}; and in the outlet section, the catalase-specific activity was 120 U mg^{-1}.

Fewer samples were analyzed for SOD and peroxidase activity than for catalase (Table 2) because the amount of crude protein extract was insufficient. As with catalase, SOD activity was highest in the inlet section of the columns. The highest observed SOD activity, 78.0 U mg^{-1}, was observed at the inlet of Column A. The SOD activity at the outlet, 24.4 U mg^{-1}, was slightly higher than that detected in the middle section, 18.5 U mg^{-1}, similar to the situation with catalase. The highest observed peroxidase activity, 0.0990 U mg^{-1}, was in the inlet of Column C, which received only 0.1 mg L^{-1} H_2O_2. Peroxidase activity in sections of Column B and A varied between 0.0282 and 0.0611 U mg^{-1}.

The mass of protein in the inlet and middle sections of Columns C (0.1 mg L^{-1} H_2O_2) and B (10 mg L^{-1} H_2O_2) was essentially the same (Table 2). The greatest difference was seen in the outlet section, where protein declined from 0.094 $\mu g\ \mu l^{-1}$ to 0.0036 $\mu g\ \mu l^{-1}$. The highest protein concentration, 0.33 $\mu g\ \mu l^{-1}$, was detected in the inlet section of Column A.

Microbial numbers

The number of viable heterotrophic microorganisms recovered on nutrient agar from Columns C and A was greatest in the inlet section and then decreased with distance from the inlet (Table 2). In Column B, the number of heterotrophs was also greatest in the inlet section; however, the numbers in all sections were less than in the inlet section of Column C. No viable microorganisms were recovered from any of the sections of the sterile column material.

In all three columns, the number of hydrocarbon-degrading microorganisms was greatest in the inlet section (Table 2). Hydrocarbon degraders in the inlet section increased over the course of the experiment, beginning at 5.6×10^5 CFU g^{-1} dry weight in Column C, going to 1.2×10^6 CFU g^{-1} dry weight in Column B, and up to 7.9×10^7 CFU g^{-1} dry weight in Column A. There was no real difference in the number of hydrocarbon degraders between the middle and outlet sections of Columns C and A and only a slight difference in Column B. A decline in heterotrophic numbers extracted from column material, but not hydrocarbon degraders, occurred after the addition of 10 mg L^{-1} H_2O_2. This increase in the percentage of hydrocarbon-degrading bacteria was one indication of a changing microbial community.

The number of heterotrophic microorganisms eluted from Columns A and B remained relatively constant during the experiment, approximately 10^6 CFU ml^{-1}. After the input BTEX concentration to Column A was increased during the addition of 250 mg L^{-1} H_2O_2, the effluent concentration of heterotrophs increased to 2.3×10^7 CFU ml^{-1}. Heterotrophs were not detected in the sterile column effluent. In Column A, the number of hydrocarbon degraders did not increase until after the input BTEX concentration had been increased on day 53.

Bacterial identification and diversity

The dominant organism in the untreated aquifer material was *Corynebacterium* sp, followed by *Pseudomonas fluor-*

Table 2 Enzyme activities, protein, and microbial numbers in column material

	Sample from	Max H_2O_2 dose received		
		0.1 mg L^{-1} (Column C)	10 mg L^{-1} (Column B)	500 mg L^{-1} (Column A)
Protein[a]	inlet	0.102	0.102	0.33
	middle	0.055	0.065	0.081
	outlet	0.094	0.0036	0.092
Catalase[b]	inlet	20.7	14.9	582
	middle	15.6	10.7	97.7
	outlet	7.29	11.0	120
SOD[b]	inlet	21.5	13.5*	78.0
	middle	ND	8.40*	18.5*
	outlet	13.4*	ND	24.4
Peroxidase[b]	inlet	0.0990*	0.0516	0.0481
	middle	ND	0.0282*	0.0611
	outlet	ND	ND	0.0420
Heterotrophs[c]	inlet	12.6	2.12	519
	middle	2.65	0.89	25.9
	outlet	0.968	1.09	16.3
Hydrocarbon degraders[c]	inlet	0.558	1.16	78.8
	middle	0.0800	0.195	6.54
	outlet	0.0791	0.144	5.92

[a]Concentration, $\mu g\ \mu l^{-1}$.
[b]Specific activity, Units mg^{-1} protein; *single SOD or peroxidase measurement.
[c]Number of CFU $\times 10^6$ g^{-1} dry weight aquifer material.
ND, not determined.

escens (Table 3). *Xanthomonas maltophilia* and *Rhodococcus equi* were less prevalent. Treatment of the subsurface material with H_2O_2 and BTEX altered the microbial community structure. The inlet of Column A was dominated by *P. fluorescens*, followed by *X. maltophilia* and *R. equi*. *X. maltophilia* was the most abundant isolate in the middle section of Column A, followed by *P. fluorescens*. The percentage of *P. fluorescens* declined from 63.6% in the column inlet to 9.3% at the outlet. *X. maltophilia* dominated the outlet section, but did not increase in percentage. *R. equi* increased from 10.3% at the inlet to 27.3% at the outlet; *Corynebacterium* sp also increased from 1.9% at the inlet to 10.4% at the outlet.

BTEX degradation

Biodegradation of BTEX began in Column C first, then A, and finally in B (Figure 2). This differential onset of biodegradation might be due to operational difficulties with Columns A and C that caused Column B to be perfused the longest before amendment with nutrients and BTEX. Interestingly, toxicity of H_2O_2, suggested by the resumed breakthrough of BTEX on day 12, was greatest in B, then A, and finally, in C. Recovery of BTEX in the sterile column remained relatively constant throughout the experiment; the average recovery for benzene, toluene, ethylbenzene, *m*-xylene and *o*-xylene was 86%, 95%, 85%, 85%, and 86%, respectively (data not shown). A clear preference for benzene, followed by toluene, as the growth substrate was apparent during H_2O_2 amendment but not prior to H_2O_2 addition (Figure 3). It was not until the addition of 10 mg L^{-1} H_2O_2 that ethylbenzene, *m*-xylene, and *o*-xylene were degraded completely in Column A.

After sacrificing Column B, which had received 10 mg L^{-1} H_2O_2, the extremely low protein concentration of the crude extracts in the outlet portion of the column suggested that the columns needed more growth substrate (Table 2). The benzene and toluene input concentrations increased from 0.5 mg L^{-1} each to 1.5 and 1.2 mg L^{-1}, respectively. During the addition of 100 mg L^{-1} H_2O_2 to Column A, the presumed enzymatic decomposition of H_2O_2 severely inpeded flow. At that time, the input concentrations of all the BTEX compounds were increased. The visible pockets of gas in the biologically active column diminished; but thenceforth, only benzene was completely

Table 3 Distribution of isolates from column material after exposure to H_2O_2 and untreated core material

Organism	Colony forming units (%)			
	Inlet	Middle	Outlet	Untreated
Pseudomonas fluorescens	63.6	32.0	9.3	33.9
Xanthomonas maltophilia[a]	18.7	44.4	44.4	2.9
Rhodococcus equi	10.3	13.3	27.3	<1
Corynebacterium sp[b]	1.9	2.9	10.4	46.8
Pseudomonas sp	4.4	7.0	5.8	13.4
Comamonas testosteroni[c]	<1	<1	<1	2.8

[a]Or *Pseudomonas maltophilia*, requires methionine.
[b]Non-pathogenic.
[c]Formerly *Pseudomonas testosteroni*, will not grow on sugars.

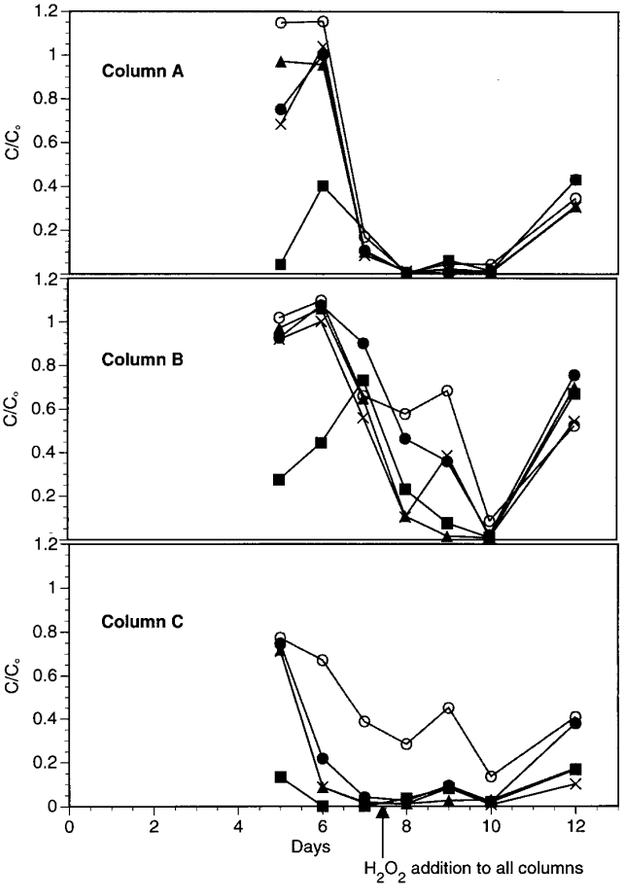

Figure 2 Biodegradation and breakthrough of BTEX after addition of 0.1 mg L^{-1} H_2O_2 for 5 days; normalized concentration computed as effluent concentration/input concentration. ■, Benzene; ●, toluene; ▲, ethylbenzene; ×, *m*-xylene; ○, *o*-xylene.

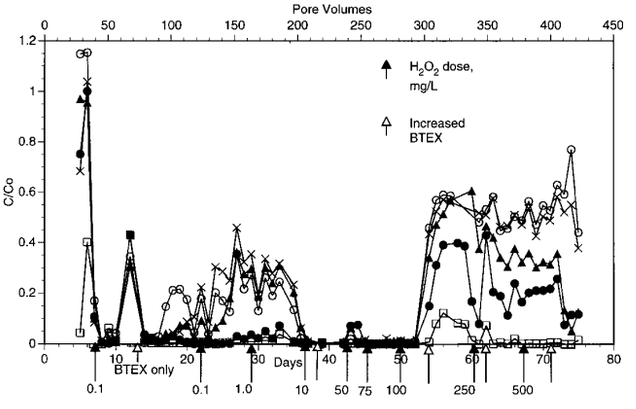

Figure 3 Biodegradation of BTEX in Column A during treatment with H_2O_2. □, Benzene; ●, toluene; ▲, ethylbenzene; ×, *m*-xylene; ○, *o*-xylene.

degraded. Because sufficient toluene, ethylbenzene, *m*-xylene, and *o*-xylene remained for degradation, the BTEX concentration was not increased when the feed solution concentration of H_2O_2 was raised to 250 mg L^{-1}. Again, pockets of gas were observed. The concentration of BTEX in the feed solution was again increased, and the off-gas-

sing was alleviated. As the H_2O_2 concentration was raised to 500 mg L^{-1}, the same events occurred, leading to another increase in the input BTEX concentration. The flow rate in Column A decreased from 2.4 ml h^{-1} to 2.0–2.2 ml h^{-1} while gas was observed. Gas entrainment and flow restriction (flow rate decreased from 2.4 ml h^{-1} to 1.35–1.6 ml h^{-1}) in the sterile column due to non-enzymatic H_2O_2 decomposition were so severe by this time that no further H_2O_2 concentration increases were possible.

The amount of BTEX extracted from column material is shown in Table 4. During the addition of 0.5 mg L^{-1} of each BTEX compound, the resulting mass added to the column material was approximately 0.5 μg g^{-1} on a daily basis. When 20 mg L^{-1} of each compound was added, the mass added was 20 μg g^{-1}. A minimal amount of BTEX mass was extracted from column material when compared with the loading of BTEX. There was no correlation between BTEX concentration and column section in the sterile control. The sterile column contained lower concentrations of toluene than the untreated starting material, Core I17. The concentrations of benzene, ethylbenzene, and *m*-xylene were greater in the sterile column extracts than in the untreated material, suggesting that sorption of these compounds may have occurred, or that the compounds may have been present in the pore water that was extracted along with the column material.

The concentrations of benzene, toluene, and ethylbenzene were lower in Column A material than in Column B or C material, which may be due to the length of time that Column A was operated. Benzene, toluene, ethylbenzene, and *m*-xylene concentrations were also lower in Column A than in any of the sections of Column S, the sterile control, suggesting that biodegradation was responsible for the reduction. The *o*-xylene concentration in Column A was less than that of the middle and outlet sections of the sterile control.

Dimethyl- and ethyl-phenols were detected by GC/MS analysis of extracts of column effluent. The 2,3-dimethyl phenol was identified in extracts of Column A effluent from day 21, before the second addition of 0.1 mg L^{-1} H_2O_2. By day 30, the 2,3-dimethyl phenol was detected along with the 3,4- and 2,4-isomers and 3-ethyl phenol. After that time, the 2,4-dimethyl phenol was the only isomer found in Column A effluent. In Column B effluent, the 2,3-isomer of dimethyl phenol was also observed before the 2,4-isomer. The 2,3-dimethyl phenol was first detected when no H_2O_2 was in the feed solution, which suggests that it was not the product of hydroxyl radical addition to *o*-xylene. However, the effluent from the sterile column was not examined for the presence of dimethyl phenols. Acetic acid, 2-methyl cyclohexanol and 2- or 4-methyl benzaldehyde were detected in the middle section of Column B material. No metabolites were detected in material from Column A or the sterile control.

Dissolved oxygen

The effluent concentration of dissolved oxygen in the biologically active columns either equaled or was less than the input concentration during the first 40 days of the experiment (Figure 4). After increasing the H_2O_2 concentration to 75 mg L^{-1} on day 45, the effluent concentration of DO was higher than the input concentration in Column A, reflecting the enzymatic and abiotic decomposition of H_2O_2. Recovery of DO in the sterile column effluent was either greater than or equal to 100% of the input DO. The effluent con-

Table 4 BTEX extracted from column material and untreated core material

Sample	Compound (μg g^{-1})					
	Benzene	Toluene	Ethyl-benzene	*m*-Xylene	*o*-Xylene	Recovery (%)
C MID 1[a]	0.456	0.286	0.444	<0.05	0.215	nd[c]
C MID 2	0.0598	<	0.128	<0.05	0.0616	nd
B MID 1	0.748	0.0800	0.403	<0.05	0.138	nd
B MID 2	0.510	0.0481	0.326	<0.05	0.113	nd
A MID 2	0.060	0.0219	0.0614	0.0988	0.134	84.1
A MID 2	0.0534	0.0384	0.0831	0.143	0.115	81.3
S IN 1[b]	0.248	0.0512	0.205	0.278	0.155	79.4
S IN 2	0.400	0.142	0.308	0.433	0.365	84.1
S IN 3	0.228	0.137	0.193	0.296	0.161	86.1
S MID 1	0.427	0.146	0.253	0.380	0.314	84.6
S MID 2	0.194	0.0675	0.173	0.294	0.210	87.5
S MID 3	0.436	0.0751	0.892	1.233	0.418	94.5
S OUT 1	0.505	0.192	0.326	0.472	0.405	84.6
S OUT 2	0.125	0.0624	0.237	0.156	0.226	81.3
S OUT 3	0.268	0.0618	0.232	0.367	0.201	77.6
Core l17[d]	<0.05	3.34	0.0912	0.0483	0.657	156
Core l17	<0.05	1.20	0.0427	0.0508	0.275	13.3

[a]Columns A, B, C sampled after receiving 500, 10, 0.1 mg L^{-1} H_2O_2 respectively.
[b]S = Sterile column; IN, MID, OUT are column inlet, midsection, and outlet.
[c]Not determined.
[d]Unamended core material used in columns.

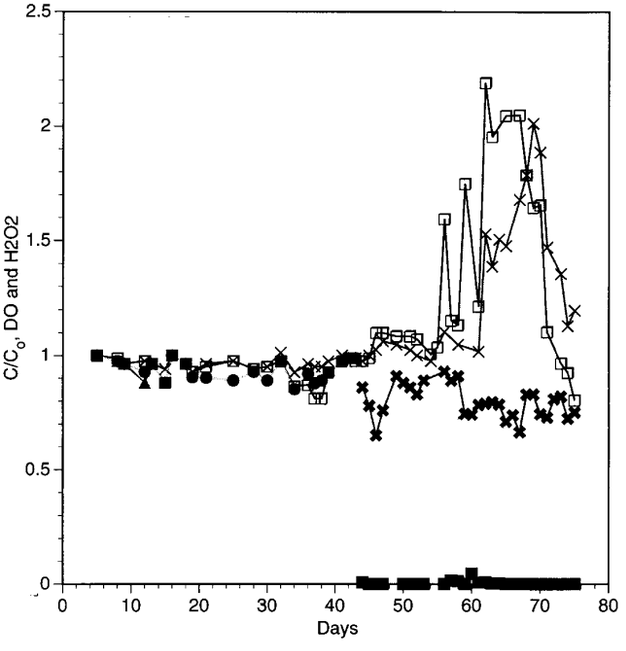

Figure 4 Recovery of dissolved oxygen (%) in biologically active columns A □, B●, and C ▲ and a sterile control, S ×, and recovery of H_2O_2 (%) in columns A ■ and S ✕.

centration was higher than the input concentration after day 60, indicating abiotic decomposition of H_2O_2.

Hydrogen peroxide

Hydrogen peroxide was not detected in the effluent of the biologically active columns. In the sterile control, an average of 79.5% of the input H_2O_2 was recovered over the course of the experiment. Figure 4 compares the effluent H_2O_2 concentration of Column A and the sterile control. During the addition of 500 mg L^{-1} H_2O_2, gas production in the sterile column was so extreme that gas pockets could be discerned and flow was disrupted. Hydrogen peroxide solution perfused through the apparatus without subsurface material in the column was completely recovered during a 2-day experiment, so the decomposition can be attributed to interaction with the aquifer material. The amount of oxygen gas produced by either mineral-catalyzed decomposition of H_2O_2 in the sterile column or catalatic plus mineral-catalyzed decomposition in the biologically active columns was not measured.

Oxygen to BTEX ratio

The cumulative masses of BTEX and oxygen consumed were determined over the specific time intervals (Table 5). The O : BTEX ratios in the three columns agreed fairly well and were lower than the stoichiometric ratio for complete mineralization and lower than what would be expected if the production of cell mass were included in the equation. The increase in ratio from 0.29–0.38 to 0.65–0.73 occurred after an increase in the oxygen mass input (10 mg L^{-1} H_2O_2). Even the overall ratio of 2.6, which is likely an overestimate, is lower than the stoichiometric ratio for complete mineralization. However, in Column A at the specific times of 250 and 500 mg L^{-1} H_2O_2 addition, the O : BTEX ratio oscillated between 2.4 and 3.8 (data not shown). This high ratio occurred in part because evolved oxygen gas was not measured, so the ratio was computed with the assumption that all H_2O_2 provided went to BTEX degradation.

Discussion

Increased enzymatic activity

The data presented here are unique in demonstrating increases in catalase and superoxide dismutase-specific activities in a mixed subsurface microbial population grown on gasoline components. Historically, induction of catalase and superoxide dismutase has been studied with pure cultures of bacteria grown on sugars or tricarboxylic acid cycle intermediates [32,41,47]. Increasing catalase activity by beginning with low initial H_2O_2 concentrations has its basis in the oxygen toxicity literature [13,62]. In this experiment, greater catalase activity was seen in comparable sections of column material as the input H_2O_2 concentration increased. Higher catalase-specific activity was also observed at the column inlet, where the highest concentration of H_2O_2 would be detected, than in the column outlet, where the H_2O_2 concentration would probably be decreased.

Although peroxidases use H_2O_2 to oxidize organic compounds, peroxidase activity did not increase in this experiment. Because the peroxidase investigated belonged to a mixed bacterial population, it is unclear whether the peroxidase activity measured was from a bifunctional hydroperoxidase such as catalase HPII, a distinct peroxidase or, perhaps, both.

SOD activity increased following H_2O_2 addition and the subsequent increase in dissolved oxygen concentration. Elevated oxygen concentration induced SOD activity in *E. coli* B and *S. faecalis* [28], in *E. coli* K12 [31], and *Campylobacter sputorum*, *Rhizobium japonium*, and *Saccharomyces cerevisiae* [29,30]. In Column C, the SOD activity of the inlet section was somewhat higher than in the outlet section, but the catalase activity was three times greater. In Column A, SOD in the inlet material was three times higher than in the outlet material, whereas catalase in the inlet was five times higher than in the outlet. Greater increases in catalase-specific activity than superoxide dismutase with H_2O_2 supplementation were expected, since H_2O_2 is a substrate of catalase but not superoxide dismutase. The increases observed in catalase activity may be attributable to a shift in the microbial population towards members with intrinsically higher catalase levels, or to the induction of catalase by specific members of the microbial population.

Microbial numbers

The number of microorganisms eluted from the columns was not a good indicator of microbial processes. The number of heterotrophs and hydrocarbon degraders fluctuated randomly (approximately 10^6 CFU ml^{-1}) prior to day 57 in Column A and in Columns B (final H_2O_2 dose, 10 mg L^{-1}) and C (final H_2O_2 dose, 0.1 mg L^{-1}). Heterotrophs and hydrocarbon degraders eluted from soil columns also varied randomly in another study of H_2O_2 supplementation [9]. The numbers of heterotrophic and hydrocarbon-degrading microorganisms eluted from Column A, the column that received the final 500 mg L^{-1} H_2O_2 dose, increased after raising the BTEX concentration in the feed solution.

The number of microorganisms extracted from column material seemed to be more indicative of microbial processes. The increased numbers of both heterotrophs and hydrocarbon degraders at the inlet section of the columns reflect stimulated growth at the column inlet. Continuous feed of a primary substrate stimulated growth at the column inlet in other studies [9,39,55]. In Column B, after receiving 10 mg L^{-1} H_2O_2, the numbers of hydrocarbon degraders were higher than in comparable sections of Column C, but the number of heterotrophs declined, suggesting either the inability to fully quantify the heterotrophic population or that the microorganisms were being selected partly on the basis of their hydrocarbon-degrading ability.

Table 5 Cumulative O : BTEX mass ratio in columns at specific time intervals

Days	Treatment	Ratio in:		
		Column A	Column B	Column C
4–12	0.1 mg L^{-1} H_2O_2	0.21	0.29	0.28
4–35	1.0 mg L^{-1} H_2O_2	0.29	0.38	
4–42	10 mg L^{-1} H_2O_2	0.65	0.73	
4–75	500 mg L^{-1} H_2O_2	2.6		

The use of artificial media for the enumeration of soil microorganisms has been documented to underestimate the total number of microorganisms present [1]. However, in one study, the number of colony forming units recovered from aquifer material constituted as much as 60% of the respiring bacteria, but the numbers of CFU and respiring bacteria were only as much as 3.5% of the total number of bacteria [42]. In a different study where cells were fractionated by filtration and each filtrate was examined by acridine orange (AO) direct counting and viable counting, the percent viability (colony forming units on agar/direct microscopic counts) in a size group was highest for the large size classes and lowest for the small cells, which led the investigators to conclude that the viable count method of enumeration allows the observation of the microorganisms that comprise the greatest biovolume [6]. Although the diversity and numbers of the microbial population were undoubtedly decreased during sample collection, storage, and growth on laboratory media, in this experiment, trends in microbial numbers extracted from column material were detected using standard plate count techniques.

Identification and distribution of bacterial isolates

Differences in microbial community structure were observed among the different segments of Column A, which had been exposed to 500 mg L^{-1} H_2O_2, and between Column A and the untreated aquifer material, Core I17, despite the fact that the diversity and numbers of bacteria reported here were likely a fraction of their *in situ* condition. The bacterial species identified in the column and untreated core materials were all soil microorganisms. Some of the bacteria are from genera that are known to include degraders of aromatic compounds. The rhodococci are able to transform xenobiotics and have been isolated from soil contaminated with petroleum [26]; they are also catalase-positive. The corynebacteria are diverse, catalase-positive, and found in soil [16]. The pseudomonads are a catalase-positive, common soil bacterial group and many are hydrocarbon degraders [43].

P. fluorescens might dominate at the column A inlet because it is more tolerant of H_2O_2, the only organism with inducible catalase, or outcompetes the other organisms for BTEX or a BTEX component. Other investigators have documented the presence of inducible catalase in a strain of *P. fluorescens* [49], but H_2O_2 was not the inducing agent. The percentage of *X. maltophilia* was higher in all sections of column material than in untreated core material. *X. maltophilia* is a degrader of BTEX, a BTEX component or metabolite; its prevalence in the presence of H_2O_2 indicates that it is tolerant to H_2O_2 and may possess an inducible catalase. Preliminary catalase induction experiments in this laboratory with the *X. maltophilia* isolate suggest that it can induce catalase-specific activity. The prevalence of *R. equi* and *Corynebacterium* sp in the material of the outlet section as compared to the inlet section imply that they were either unable to increase catalase levels, less tolerant to H_2O_2, or not as successful in exploiting certain BTEX components as were *P. fluorescens* and *X. maltophilia*. The enrichment of *R. equi* in Column A compared to the untreated material suggests that it may be tolerant to H_2O_2, able to increase catalase, or degrade at least one of the BTEX compounds. *R. equi* was isolated from hydrocarbon-degrading medium, supporting the latter possibility. The lack of detection of *Comamonas testosteroni* in the column material and the decline of *Corynebacterium* sp from its dominance in the untreated material indicate that they were unable to tolerate or adapt to H_2O_2. The elimination of one bacterial species and the reduction of another species signify changes that were caused by the continuous addition of H_2O_2 and BTEX.

BTEX biodegradation

Following the addition of H_2O_2, benzene was completely degraded throughout the course of the experiment. Toluene was the next most completely degraded compound, followed by ethylbenzene and the *m*- and *o*-xylene isomers. Some studies indicate that toluene is more readily degraded than benzene [36,58] with the time for complete removal increasing from toluene $<$ benzene $=$ *p*-xylene $<$ *o*-xylene [25]. Before the introduction of H_2O_2 that presumably changed the composition of the microbial community, more *m*-xylene than *o*-xylene was degraded. A preference for the *meta* isomer of xylene in aerobic biodegradation was observed in several other studies [38,58].

BTEX components can act cooperatively in biodegradation. The presence of either toluene or *o*-xylene was shown to stimulate the biodegradation of benzene by a mixed culture, but when toluene and *o*-xylene were present together, their stimulation was not as great [4]. Other investigators have noted inhibited degradation of some BTEX components in the presence of the other components. The presence of *p*-xylene increased the lag period for the degradation of benzene but did not affect its rate of biodegradation; *p*-xylene increased the lag period and decreased the rate of biodegradation of toluene in microcosms containing aquifer slurries; the rate of *p*-xylene degradation was greatest while toluene was simultaneously degraded [2].

The degradation intermediates observed in this research have been documented as intermediates in aerobic and anaerobic metabolism. Methyl and ethyl phenols, along with organic acids, were identified along with aromatic pollutants in leachate from a landfill site [52]. The methyl phenol isomers are intermediates in the anaerobic degradation of toluene [27], but they can also occur in aerobic metabolism [24,53]. The methyl cyclohexanol might be the product of *o*-hydroxylation of toluene followed by a ring reduction. A similar sequence of reactions is proposed in the anaerobic degradation of toluene by mixed methanogenic cultures [27]. Oxidation of one of the methyl groups of *o*-xylene would yield 2-methyl benzaldehyde.

During the time period (day 20–30) in which 2,3-dimethyl phenol was detected in column effluent, more *o*-xylene than *m*-xylene was degraded (Figure 3). The occurrence of 2,3-dimethyl phenol is likely from an *ortho* hydroxylation of the aromatic ring of *o*-xylene. Under aerobic conditions, Shields *et al* [53] observed *ortho* hydroxylation of toluene leading to the formation of 2-methyl phenol, followed by a *meta* hydroxylation that yielded 3-methyl catechol. *Meta* hydroxylation of *o*-xylene could result in the formation of 3,4-dimethyl phenol; *meta* hydroxylation of ethylbenzene might yield 3-ethyl phenol.

The 2,4-dimethyl phenol could be the result of a *para* hydroxylation of *m*-xylene. *Para* hydroxylation of toluene

occurs in *Pseudomonas mendocina* under aerobic conditions [24]. The 2,4-dimethyl phenol might also have arisen from an *ortho* hydroxylation, this time of *m*-xylene. The data suggest that the ethyl- and dimethyl-phenols are metabolic intermediates, but lack of comparison with the sterile column effluent allows the possibility that the dimethyl phenols were the product of an abiotic reaction.

The theoretical, initial ratio of oxygen to BTEX mass provided to the columns was 3.25 : 1, assuming mineralization. In the early part of this experiment, the ratio of oxygen to BTEX mass consumed was almost an order of magnitude less than the ratio for complete mineralization, and one-third to one-half the ratio expected if cell growth is considered in the equation [12]. Later in the experiment, the O : BTEX ratio increased. The increase could be the result of stimulating the bacteria or enzymatic and abiotic decomposition of the H_2O_2 without use of the oxygen evolved. Although the 0.3 ratio observed initially in this work may be site-specific (owing to the biomass concentration and substrate concentration), it suggests that extreme conservatism may occur in the design of *in situ* bioremediation projects, by supplying more oxygen than necessary for aerobic biodegradation.

Over the entire experiment, the ratio of oxygen to BTEX consumed was 2.6. In other studies of H_2O_2-supplemented biodegradation, the observed oxygen to hydrocarbon mass ratio ranged from 4.43 to 5.23 [35] and 8.1 to 10.3 [7]. The high mass ratio reported by Barenschee *et al* [7] was attributed to an increase in biomass and degassing of H_2O_2. In our research, high ratios of oxygen to BTEX mass utilization were observed during periods of H_2O_2 decomposition.

Eliminating the off-gassing and flow problems associated with catalatic, and possibly abiotic, decomposition of H_2O_2 by increasing the BTEX input concentration has implications that are important to the practice of *in situ* bioremediation supplemented with H_2O_2 and might explain the phenomena observed in other laboratory and field experiments. In a possible scenario, the availability (or continuous supply) of a carbon and energy source provides both the means for synthesis of oxygen defense enzymes and a sink for the oxygen evolved by those enzymes. Lack, or depletion, of a carbon and energy source might render microorganisms unable to protect themselves against the toxicity of H_2O_2. Decreased viable counts were observed in the deeper zones at Traverse City [21], similar to the decreased heterotrophic numbers and protein concentration in Column B, which had received increased H_2O_2 ($10\ mg\ L^{-1}$) without an increase in BTEX concentration. Increased catalase and minimal toxicity were seen in the shallow contaminated zones at Traverse City [21]; the shallow zone could be considered to have a continuous carbon source because infiltration released the more soluble components of aviation gasoline from the capillary fringe into the aquifer [11]. In a field study at Eglin Air Force Base [19], bacterial decomposition of H_2O_2 in the infiltration gallery coincided with the use of recirculated ground water containing residual JP-4 jet fuel. When the carbon and energy source was removed by using tap water in the infiltration gallery, rather than recirculated ground water, the added H_2O_2 was stable [57]. A carbon-limited stage was also identified in another study of H_2O_2-supplemented biodegradation [7].

Although this research focused on increased catalase-specific activity as an indication of tolerance and adaptation to H_2O_2, several other mechanisms may be important in increased tolerance to H_2O_2, such as the induction of DNA repair enzymes, formation of thicker biofilms, differences in cell wall and capsule materials, and non-enzymatic scavenging by intracellular molecules. In environments where only slight contamination exists, the toxicity of H_2O_2 is evident. If enough substrate is initially available for the synthesis of oxygen defense enzymes and the development of other protective mechanisms, continued application of H_2O_2 will almost certainly lead to the production of oxygen. The oxygen produced can then be eliminated by fulfilling the carbon and energy demand that the excess oxygen requires, or the problem can be solved by removing the biological activity.

Addition of H_2O_2 in increasing doses to the subsurface, while useful in allowing microorganisms to adapt to H_2O_2, may be counterproductive if the available concentration of hydrocarbon does not also increase. One may begin to see increases in DO concentration, as during this experiment when the input BTEX concentration was insufficient for consumption of the oxygen produced through catalatic (and abiotic) action. An elevation of DO might be considered an indication that the H_2O_2 concentration is too high, because any available oxygen should be used for biodegradation if one assumes instantaneous reaction kinetics for the reduction of oxygen.

The anticipated decrease in completion time for an *in situ* bioremediation project may not justify the potential toxicity and subsequent change in the microbial community structure associated with H_2O_2 addition. Supplementation with H_2O_2, rather than decreasing the time to completion of a project, could jeopardize the successful biodegradation of target compounds if the microorganisms responsible for degradation are unable to tolerate H_2O_2. In this research, considerable change occurred in the distribution of the bacterial community after H_2O_2 addition. Perhaps the incomplete degradation of toluene, ethylbenzene, *m*-xylene, and *o*-xylene can be attributed to the diminished presence of efficient degraders of these chemicals. The perceived advantage of H_2O_2 addition could be offset by the selection of more H_2O_2-tolerant organisms that are not necessarily more efficient degraders. Considering the gas production and toxicity that have been associated with H_2O_2, in this case it can safely be stated that more H_2O_2 was not better.

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