

FINAL REPORT- Phase II

Novel Microbially-Driven Fenton Reaction for In Situ Remediation
of Groundwater Contaminated With 1,4-Dioxane,
Tetrachloroethene (PCE) and Trichloroethene (TCE)

SERDP Project ER-2305

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14. ABSTRACT Hazardous contaminants of heightened concern at DoD sites include the chlorinated solvents tetrachloroethene and trichloroethene, the solvent stabilizer 1,4-dioxane, and perfluoroalkyl substances. Recent concern over these contaminants in surface waters and subsurface aquifers is driven by several factors, including widespread use and improper disposal practices by industry, high miscibility in water, recalcitrance to conventional degradation processes, and classification as probable human carcinogens. Current remediation technologies entail ex situ pump-and-treat procedures that are neither cost effective nor able to effectively remove co-contaminants such as 1,4-dioxane, tetrachloroethene, and trichloroethene, and perfluorooctanoic acid. The main objective of this work was to develop alternative ex situ and in situ bioremediation technologies based on a microbially driven Fenton reaction for degradation of hazardous contaminants at DoD sites.						
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List of Acronyms

AFFF	Aqueous film-forming foam
AOP	Advanced oxidative process
DD	Dioxane degrading
DIC	Dissolved inorganic carbon
DNA	Deoxyribonucleic acid
DOD	Department of Defense
DTPA	Diethylenetriaminepentaacetic acid
FAD	Flavin adenine dinucleotide
FIA	Flow injection analysis
FMN	Flavin mononucleotide
FTC	Flow through column
HBC	Hydroxy benzoic acid
HEAA	Hydroxyethoxyacetic acid
HPLC	High pressure liquid chromatography
IC	Ion chromatography
LB	Lysogeny broth
LC-MS/MS	Liquid chromatography combined with tandem mass spectrometry
LS	Low salt
MTBE	Methyl tert-butyl ether
NADPH	Nicotinamide adenine dinucleotide phosphate
nZVI	Nanoparticulate zero-valent iron
PCE	Perchloroethylene (tetrachloroethylene)
PCP	Pentachlorophenol
PCR	Polymerase chain reaction
PFAS	Perfluoroalkyl substance
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonic acid
ROS	Reactive oxygen species
TCA	1,1,2-trichloroethane
TCE	Trichloroethylene
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
UV	Ultraviolet
XRD	X-ray diffraction

Keywords

Microbially-driven Fenton reaction

Hydroxyl radical

Shewanella oneidensis MR-1

Trichloroethylene

Tetrachloroethylene

Perchloroethylene

1,4-dioxane

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Abstract

A. Introduction and Objectives. Hazardous contaminants of heightened concern at DOD sites include the chlorinated solvents tetrachloroethene and trichloroethene, the solvent stabilizer 1,4-dioxane, and perfluoroalkyl substances. Recent concern over these contaminants in surface waters and subsurface aquifers is driven by several factors, including widespread use and improper disposal practices by industry, high miscibility in water, recalcitrance to conventional degradation processes, and classification as probable human carcinogens. Current remediation technologies entail *ex situ* pump-and-treat procedures that are neither cost effective nor able to effectively remove co-contaminants such as 1,4-dioxane, tetrachloroethene, and trichloroethene, and perfluorooctanoic acid. The main objective of this work was to develop alternative *ex situ* and *in situ* bioremediation technologies based on a microbially driven Fenton reaction for degradation of hazardous contaminants at DOD sites.

B. Technical Approach. The microbially driven Fenton reaction was subsequently employed to drive a HO[•] radical-generating Fenton reaction that degraded 1,4-dioxane, tetrachloroethene, and trichloroethene. In the microbially driven Fenton degradation system, *S. oneidensis* batch cultures were provided with lactate as electron donor and Fe(III) as anaerobic electron acceptor and exposed to alternating aerobic-anaerobic conditions. During the aerobic phase, *S. oneidensis* reduced oxygen to hydrogen peroxide, while during the anaerobic phase *S. oneidensis* reduced Fe(III) to Fe(II). During the aerobic-to-anaerobic transition period, the produced Fe(II) and hydrogen peroxide interacted chemically via the Fenton reaction to form Fe(III), HO⁻ ion, and HO[•] radical which in turn can degrade contaminants. This approach was tested in liquid batch reactors, fed-batch reactors, and solid-state flow-through reactors. Further manipulation of Fenton reaction rates was examined by deleting reactive oxygen species (ROS) related genes in *S. oneidensis*. H₂O₂ is a byproduct of incomplete metabolism of oxygen during aerobic respiration, and a key reactant during the Fenton reaction. By manipulating select genes, including catalases and peroxidases directly implicated in ROS defense in *S. oneidensis*, we endeavor to enhance the rate of contaminant degradation.

C. Results. During the aerobic-to-anaerobic transition period, the microbially-driven Fenton reaction oxidatively degraded source-zone concentrations of 1,4-dioxane, tetrachloroethene, and trichloroethene. This method was applied to attempt degradation of perfluorooctanoic acid, but it remained recalcitrant to the microbially-driven Fenton reaction. Batch reactor experiments demonstrated that 1,4-dioxane degradation by the microbially-driven Fenton reaction may not be as efficient with ferrihydrite than ferric citrate as terminal electron acceptor. As organic ligands are known to affect the Fenton reaction, it is likely that stabilization of Fe(II) by ligands in solution as well as participation of the organic ligand in dark ROS production affects the generation of hydroxyl radicals that are involved in organic contaminant degradation. Furthermore, comparison of *S. oneidensis* H₂O₂ scavenging with ROS mutants and other *Shewanella* species demonstrates *S. oneidensis* as the weakest H₂O₂ scavenger tested. Perhaps the ability of *S. oneidensis* to degrade contaminants is due to poor ROS scavenging causing excessive extracellular hydroxyl radicals.

D. Benefits. The microbially driven Fenton reaction provides a foundation for development of alternate *ex situ* and *in situ* remediation technologies to degrade contaminants DOD sites. In addition, the microbially driven Fenton reaction may be applied as an alternate remediation technology for a broad range of other emerging contaminants susceptible to degradation by hydroxyl radicals generated by the Fenton reaction, and thus may benefit remediation efforts throughout the United States.

Executive Summary

Introduction. Heightened concern over 1,4-dioxane in contaminated surface and ground waters at DOD sites is driven by several factors, including the extensive use and improper disposal of 1,4-dioxane in industrial processes, the high mobility of 1,4-dioxane in water, the recalcitrance of 1,4-dioxane to degradation in the environment, and the classification of 1,4-dioxane as a probable human carcinogen. Current 1,4-dioxane remediation technologies generally entail *ex situ* pump-and-treat procedures that are neither cost effective nor able to remove co-contaminants tetrachloroethene (PCE), trichloroethene (TCE) and perfluoroalkyl substances (PFAS) such as the flame retardant additives perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA). Cost-effective remediation technologies for *ex situ* and *in situ* removal of 1,4-dioxane and co-contaminants PCE, TCE, and PFAS have yet to be fully developed. Bioremediation is a promising alternative method for in situ remediation of 1,4-dioxane- and other contaminated waters. Contaminants may be degraded microbially via metabolic or cometabolic reactions. Microbial degradation is carried out aerobically by mixed microbial communities in industrial sludge, and 1,4-dioxane-degrading bacteria have been isolated. Only a limited number of studies have examined microbial degradation of 1,4-dioxane under anaerobic conditions, and the degradation rates under nitrate-, iron-, and sulfate-reducing conditions are exceedingly slow. *Ex situ* treatment of 1,4-dioxane-contaminated ground water involves pumping and treatment via advanced oxidation processes.

In the Fenton reaction (equation 1), H₂O₂ reacts with ferrous iron (Fe(II)) to produce ferric iron (Fe(III)), hydroxyl ion (OH⁻), and hydroxyl radical (HO[•]):



Due to their high oxidation potential, Fenton reaction-generated HO[•] radicals oxidatively degrade a wide variety of hazardous organic compounds, including landfill leachates, chlorinated aliphatics and aromatics, dry-cleaning solvents, pharmaceuticals, pentachlorophenol (PCP), tetrachloroethene (PCE), trichloroethene (TCE) and 1,1,2-trichloroethane (TCA).

Microbially driven Fenton reactions based on production of H₂O₂ via microbial O₂ respiration and Fe(II) via microbial Fe(III) reduction alleviate the need for continual addition of H₂O₂ and Fe(II) that drive the chemical Fenton reaction. The microbially driven Fenton reaction designed in the present study was based on the original observation that the Fe(III)-reducing facultative anaerobe *Shewanella oneidensis* liquefied the agar support directly beneath colonies grown aerobically on solid growth medium supplemented with Fe(III). Under such conditions, high microbial O₂ consumption rates directly beneath the colony effectively lowered the O₂ concentrations to levels that permitted simultaneous microbial Fe(III) reduction, concomitant production of the Fenton substrates H₂O₂ and Fe(II), and agar liquefaction via interaction with the resulting HO[•] radicals. The microbially driven Fenton reaction was subsequently employed to drive a HO[•] radical-generating Fenton reaction that degraded pentachlorophenol (PCP), a highly toxic chlorinated compound also widely distributed in contaminated ground waters and subsurface aquifers. In the PCP degradation system, *S. oneidensis* batch cultures were provided with lactate as electron donor and Fe(III) as anaerobic electron acceptor and exposed to alternating aerobic-anaerobic conditions. During the aerobic phase, *S. oneidensis* reduced O₂ to H₂O₂, while during the anaerobic phase *S. oneidensis* reduced Fe(III) to Fe(II). During the aerobic-to-anaerobic transition period, the produced Fe(II) and H₂O₂ interacted chemically via the Fenton reaction to form Fe(III), OH⁻ ion, and HO[•] radical, which, in turn, oxidatively dechlorinated PCP. This microbially driven Fenton degradation system was autocatalytic since continual inputs of Fe(II)

and H₂O₂ were not required to drive degradation. Fe(III) produced by H₂O₂-catalyzed Fe(II) oxidation was readily re-reduced back to Fe(II) by *S. oneidensis* in subsequent anaerobic phases.

Objectives. Based on the ability of the *S. oneidensis*-driven Fenton reaction to catalyze the oxidative dechlorination of PCP without continual addition of Fenton reagents Fe(II) or H₂O₂, we proposed that the system also catalyzes the degradation of 1,4-dioxane, PCE, and TCE. The main objectives of the Project ER-2305 were to i) design a microbially driven Fenton reaction that autocatalytically generated HO• radicals and degraded 1,4-dioxane, TCE, and PCE singly or in combination as co-contaminants at circumneutral pH without the need for continual addition of H₂O₂ or UV irradiation to regenerate Fe(II), ii) optimize the 1,4-dioxane degradation rates by varying the duration and frequency of the aerobic and anaerobic incubation periods, and iii) determine the pathway for 1,4-dioxane degradation by identifying the transient intermediates produced during the microbially driven Fenton reaction for 1,4-dioxane degradation.

Upon completion of the above, the project expanded to include the chemicals TCE and PCE. Although TCE and PCE are often comingled with 1,4-dioxane in contaminated ground water, only a limited number of studies have examined simultaneous degradation of multiple contaminants, with emphasis on binary mixtures of TCE and 1,4-dioxane or TCE and PCE. The main objectives of the present study were to (i) design a new fed batch, microbially-driven Fenton reaction system that minimizes contaminant loss due to volatility by separating the Fe(II)-generating, H₂O₂-generating, and contaminant degradation phases and ii) apply the new microbially-driven Fenton reaction system to simultaneously degrade single, binary, and ternary mixtures of TCE, PCE, and 1,4-dioxane.

Upon completion of the above, we applied the microbially driven Fenton reaction system to the emerging contaminant perfluorooctanoic acid (PFOA). The objective of this research was to develop novel ex situ and in situ remediation technologies for degradation of PFAS, which have received recent heightened attention as emerging contaminants and persistent organic pollutants. In this component of the project, PFOA degradation was examined via the microbially-driven Fenton reaction. However, PFOA ultimately proved to be recalcitrant to this approach due to apparent lack of reactivity with the HO• radical.

As iron oxyhydroxides and iron-reducing bacteria are ubiquitous in soils and sediments, the microbially-driven Fenton reaction may represent an economical in situ bioremediation strategy for organic contaminants. This study used flow-through and batch incubations to: (i) determine whether natural Fe(III) oxyhydroxide substrates promote the microbially-driven Fenton process; (ii) determine whether the microbially-driven Fenton reaction process and the degradation of 1,4-dioxane as example of contaminant can be sustained in flow-through columns (FTCs); and (iii) study the mechanism of the reaction and optimize experimental conditions to make the microbially-driven Fenton reaction sustainable in FTCs, with the ultimate goal of transferring this technology to pump-and-treat applications.

As demonstrated, microbial production of extracellular H₂O₂ can be exploited in engineered systems to drive the Fenton reaction (Eq. 1) for degradation by oxidation of recalcitrant compounds that would otherwise persist in the environment. Other reactive oxygen species (ROS) include peroxides and superoxide, which are byproducts of incomplete metabolism of molecular oxygen during cell metabolism and extracellular processes. By manipulating select genes directly implicated in defense against oxidative stress in the facultative anaerobe *Shewanella oneidensis*, including catalases ($2 \text{ H}_2\text{O}_2 \rightarrow 2 \text{ H}_2\text{O} + \text{O}_2$) and peroxidases ($\text{ROOR}' + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{ROH} + \text{R}'\text{OH}$), we hope to enhance the treatment system/process by increasing rate of the Fenton reaction and thereby the rate of contaminant degradation.

Technical Approach. *Design of a Microbially Driven Fenton Reaction for 1,4-Dioxane Degradation in a Batch Reactor System.* The experimental conditions for the microbially-driven Fenton degradation of 1,4-dioxane consisted of *S. oneidensis* batch cultures amended with Fe(III) and 1,4-dioxane, and exposed to different aerobic/anaerobic cycling periods. *S. oneidensis* was grown aerobically in LB on a rotary shaker (200 rpm, 30 °C) to early stationary phase ($OD_{600} = 1.5$), harvested by centrifugation at 6000 x g, washed and resuspended in LS medium to a final cell density of 10^9 cells per ml. Anaerobic stock solutions of Fe(III) citrate and 1,4-dioxane were added to final concentrations of 10 mM each. The cell culture was allowed to reduce Fe(III) citrate for pre-selected time periods (45 min, 1.5 h, 3 h, and 6 h) under anaerobic conditions maintained by continuously sparging with hydrated high-purity nitrogen to determine optimal cycle time. Reactor temperature (25°C) and pH (7.0) were held constant in all experiments. Aerobic conditions were initiated by sparging the culture with hydrated compressed air for pre-selected time periods (45 min, 1.5 h, 3 h, and 6 h). Cell density was monitored by determining colony forming units on LB agar plates incubated at 30 °C for 72 h. Analytical methods can be found in (Sekar et al., 2014).

Design of Fed Batch Reactor System for Simultaneous Degradation of TCE, PCE, and 1,4-Dioxane by the Microbially-Driven Fenton Reaction. The toxicity of single, binary, and ternary mixtures of TCE, PCE, and 1,4-dioxane was tested in LS medium by comparing aerobic growth of *S. oneidensis* batch cultures in the presence and absence of the contaminant mixtures. To avoid inadvertent loss of contaminants due to volatility during injection of compressed nitrogen or compressed air, the batch reactor system previously employed for 1,4-dioxane degradation was modified and the Fe(II)-generating, H_2O_2 -generating, and contaminant degradation phases were separated. In the Fe(II)-generating phase, contaminant-free *S. oneidensis* liquid cultures (10^9 cells mL^{-1} in LS medium amended with 10 mM Fe(III)) were incubated in 60-mL glass serum bottles under anaerobic conditions by injecting (hydrated) compressed nitrogen until the entire 10 mM pool of Fe(III) was reduced to approximately 10 mM Fe(II) (Fe(II)-generating phase). The compressed nitrogen line was then replaced by a (hydrated) compressed air line and the 10 mM Fe(II)-containing *S. oneidensis* liquid culture was incubated under aerobic conditions until the 10 mM pool of Fe(II) was oxidized to approximately 4 mM level (H_2O_2 -generating phase). The 4 mM residual Fe(II) was carried into the contaminant degradation phase to interact with the pool of microbially-produced H_2O_2 and generate $HO^\bullet$ radicals. The contaminant degradation phase was initiated by adding single, binary, and ternary mixtures of TCE, PCE, and 1,4-dioxane with a sterile syringe. The contaminant degradation phase was carried out for approximately 5 days (without gas injection) and the contaminant concentrations were monitored via high pressure liquid chromatography (HPLC). Two similar cycles were repeated. Analytical methods can be found in (Sekar et al., 2016).

Design of a Microbially Driven Fenton Reaction for Perfluorooctanoic Acid in a Batch Reactor System. The experimental conditions for the microbially-driven Fenton degradation of PFOA were nearly identical to the batch system designed to degrade dioxane. Each bioreactor consisted of *S. oneidensis* batch cultures amended with Fe(III) and 1 μ M PFOA, and exposed to an aerobic/anaerobic cycling period of 3 hours. Aerobic conditions were initiated by sparging the culture with hydrated compressed air for pre-selected time periods and cycled four times. Immediately following decommissioning of batch reactors, 2 mL of liquid culture samples were aliquoted into microcentrifuge tubes. Each sample was spiked with ^{13}C labeled M2PFOA (extraction control) for a final concentration of 100nM and stored at 4C. 3 mL of HPLC-grade water (pH=10, adjusted with NaOH) was added to 0.5 mL of liquid culture in a 15 mL borosilicate glass tube, then vortexed on high speed for 10 seconds and incubated at room temperature for 30

minutes. 4 mL of methyl tert-butyl ether (MTBE) was added to the tube and vortexed on high for 20 minutes. Tubes were set upright to allow separation of aqueous and organic layers, then MTBE (organic layer containing PFOA) was removed using a glass syringe with a steel luer-lock needle to a new 15mL borosilicate glass tube. MTBE extraction steps were repeated three times, then stored at 4C if required. Samples were centrifuged in a speedvac at 4000rpm for 1.5 hrs to evaporate MTBE from tubes, then resuspended in 1mL of 90:10 H₂O:acetonitrile. Each sample was spiked with ¹³C labeled M8PFOA (instrument control) for a final concentration of 100nM. Samples were vortexed and transferred to HPLC vials, then analyzed using liquid chromatography combined with tandem mass spectrometry (LC-MS/MS). The full analytical procedure can be found in the extended final report.

Design of a Microbially Driven Fenton Reaction for 1,4-Dioxane Degradation in a Flow-Through Reactor System. FTCs consist of cylindrical reactors of 15 cm long and 4 cm diameter that are filled with freshly prepared ferrihydrite-coated sand. The FTCs include a flow cell positioned in line with the outlet stream that can accommodate voltametric microelectrodes to measure the composition of the outlet stream as a function of time. Outlet streams are sampled after the flow cell with a fraction collector for analysis of reactants and products. Using voltametric Au/Hg microelectrodes with a potentiostat and multiplexer that is operated autonomously, the concentration of dissolved oxygen (O_{2(aq)}) and Fe(II) are monitored at each electrode simultaneously in the output flow. Additional analyses conducted in the outlet fluids include: inorganic anions and organic acids by ion chromatography; H₂O₂ by horseradish peroxidase oxidation with resorufin as fluorescent indicator after stabilization in 100 mM diethylenetriaminepentaacetic acid (DTPA) to inhibit iron redox rxns; and dioxane by HPLC.

Enhancement of the Microbially Driven Fenton Reaction via Genes Involved in Oxidative Stress Response. Ten in-frame single and double gene deletion mutants related to oxidative stress were constructed in *Shewanella oneidensis* MR-1. One dedicated catalase (SO_1070, $\Delta katB$), two bifunctional catalase-peroxidases (SO_0725, $\Delta katG2$; SO_4405, $\Delta katG1$), and periplasmic glutathione peroxidase (SO_3349, $\Delta pgpD$) were deleted from *S. oneidensis* MR-1 as described in (Toporek *et. al.*, 2019). Double deletion combinations of each gene were also constructed to further elucidate the effects of each gene on ROS defense. To compare the abilities of the *Shewanella* spp. to remove H₂O₂ from their environment, strains were seeded overnight at 30°C in lysogeny broth, harvested by centrifugation at 3000 rpm, washed and transferred into minimal medium amended with 20mM lactate at an 2×10^7 cells mL⁻¹. Cells were incubated at 30°C until harvesting at mid-log phase, washed twice, then inoculated at 5×10^7 cells mL⁻¹ into a 24-well plate holding 2mL minimal medium amended with 20mM lactate and 0-100 μ M of H₂O₂. Samples were collected every 3-5 minutes and analyzed immediately for residual exogenous H₂O₂ using the resorufin-horseradish peroxidase colorimetric assay. Plates were incubated aerobically at room temperature with shaking for the duration of the experiments (30-200min).

Results and Discussion. *Microbially Driven Fenton Reaction for Degradation of the Widespread Environmental Contaminant 1,4-Dioxane.* The 1,4-dioxane degradation process was driven by pure cultures of the Fe(III)-reducing facultative anaerobe *Shewanella oneidensis* manipulated under controlled laboratory conditions. *S. oneidensis* batch cultures were provided with lactate, Fe(III), and 1,4-dioxane and were exposed to alternating aerobic and anaerobic conditions. The microbially driven Fenton reaction completely degraded 1,4-dioxane (10 mM initial concentration) in 53 h with an optimal aerobic-anaerobic cycling period of 3 h. Acetate and oxalate were detected as transient intermediates, an indication that conventional and microbially-driven Fenton degradation processes follow similar reaction pathways.

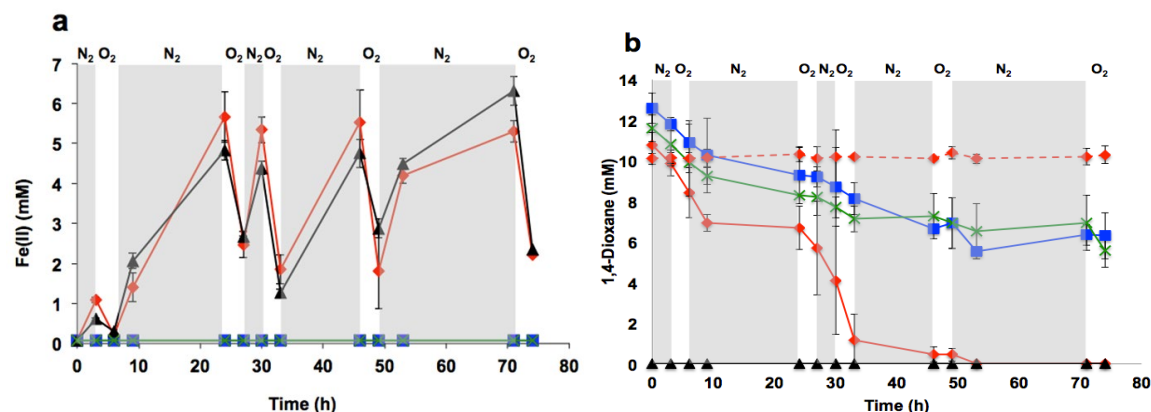


Figure 1. Fe(II) and 1,4-dioxane concentrations during microbially-driven Fenton degradation of 1,4-dioxane (10 mM initial concentration) with an aerobic/anaerobic cycling period of 3 h: (a) Fe(II); (b) 1,4-dioxane; red $\blacklozenge$, cells + 1,4-dioxane + Fe(III); blue $\blacksquare$, *S. oneidensis* cells omitted; black $\blacktriangle$, 1,4-dioxane omitted; green $\times$, Fe(III) omitted; dashed red $\blacklozenge$, No Gas control. Grey shaded areas correspond to anaerobic phases and unshaded areas correspond to aerobic phases.

To initiate $\text{HO}^\bullet$ radical production by the *S. oneidensis*-driven Fenton reaction, Fe(III)-containing *S. oneidensis* cultures were exposed to alternating and anaerobic conditions. During the aerobic phase, *S. oneidensis* reduced O_2 to H_2O_2 , while during the anaerobic phase, *S. oneidensis* reduced Fe(III) to Fe(II) (Fig. 1a). During the aerobic-to-anaerobic transition period, the produced Fe(II) and H_2O_2 interacted chemically via the Fenton reaction to form Fe(III), OH^- ion, and $\text{HO}^\bullet$ radical which, in turn, degraded 1,4-dioxane at source zone concentrations (6 mg/L, 10 mM) (Fig. 1b). Subsequent optimization studies demonstrated that 10 mM 1,4-dioxane was degraded to below detection limits in 53 h with optimum aerobic-anaerobic cycling periods of 3 h. The 1,4-dioxane degradation system was autocatalytic since continual inputs of Fe(II) and H_2O_2 were not required to drive 1,4-dioxane degradation. Fe(III) produced by H_2O_2 -catalyzed Fe(II) oxidation was readily re-reduced back to Fe(II) by *S. oneidensis* in subsequent anaerobic phases. These results demonstrate that the *S. oneidensis*-driven Fenton system degrades 1,4-dioxane without continual addition of Fenton reagents Fe(II) and H_2O_2 .

Concentrations of 1,4-dioxane remained constant in the presence of the $\text{HO}^\bullet$ radical-scavenging compounds, thus indicating that $\text{HO}^\bullet$ radicals are involved in the microbially driven 1,4-dioxane degradation process (not shown). The requirement for microbial Fe(III) reduction was tested by replacing Fe(III) with NO_3^- and carrying out an otherwise identical set of incubations with NO_3^- -containing *S. oneidensis* cultures subjected to alternating aerobic and anaerobic cycling periods. In these experiments, 1,4-dioxane was not degraded with NO_3^- as electron acceptor, nor was 1,4-dioxane degraded in the absence of Fe(III) or *S. oneidensis* cells. These results indicate that microbial Fe(III) reduction was required to continually drive the 1,4-dioxane degradation process.

TCE Degradation in the Presence and Absence of PCE and 1,4-Dioxane. Rates of degradation of TCE as sole contaminant were 18-54% lower in binary and ternary mixtures of PCE and 1,4-dioxane (Fig. 4a). Decreases in TCE degradation rates in the binary and ternary contaminant mixtures are most likely due to competition for $\text{HO}^\bullet$ radicals by PCE (with a $k_{\text{HO}^\bullet}$ 62% higher than TCE) or 1,4-dioxane (with a $k_{\text{HO}^\bullet}$ approximately 43% higher than TCE and amended at 20-fold higher concentrations than TCE). Rates of degradation of PCE as sole contaminant were not affected by TCE in binary mixtures of TCE and PCE, but were 4-38 % lower in binary and ternary mixtures containing 1,4-dioxane. The inability of TCE to affect PCE degradation rates is

most likely due to the 62% higher $k_{HO^\bullet}$ for PCE than TCE. Decreases in PCE degradation rates in the binary and ternary contaminant mixtures containing 1,4-dioxane are most likely due to competition for $HO^\bullet$ radicals by 1,4-dioxane, which was amended at 20-fold higher concentrations than PCE. Rates of degradation of 1,4-dioxane as sole contaminant were 39-65% lower in binary and ternary mixtures of TCE and PCE (Fig 2). Decreases in 1,4-dioxane degradation rates in the binary and ternary contaminant mixtures are most likely due to competition for $HO^\bullet$ radicals by PCE (with a $k_{HO^\bullet}$ 13% higher than 1,4-dioxane; Fig. 4c). During the third 1,4-dioxane degradation cycle, however, 1,4-dioxane degradation rates rebounded to 70 – 101% of the degradation rates with 1,4-dioxane as sole contaminant, most likely due to decreases in PCE concentrations below threshold levels for PCE to compete effectively with 1,4-dioxane for $HO^\bullet$ radicals during the third contaminant degradation cycle (Fig. 2).

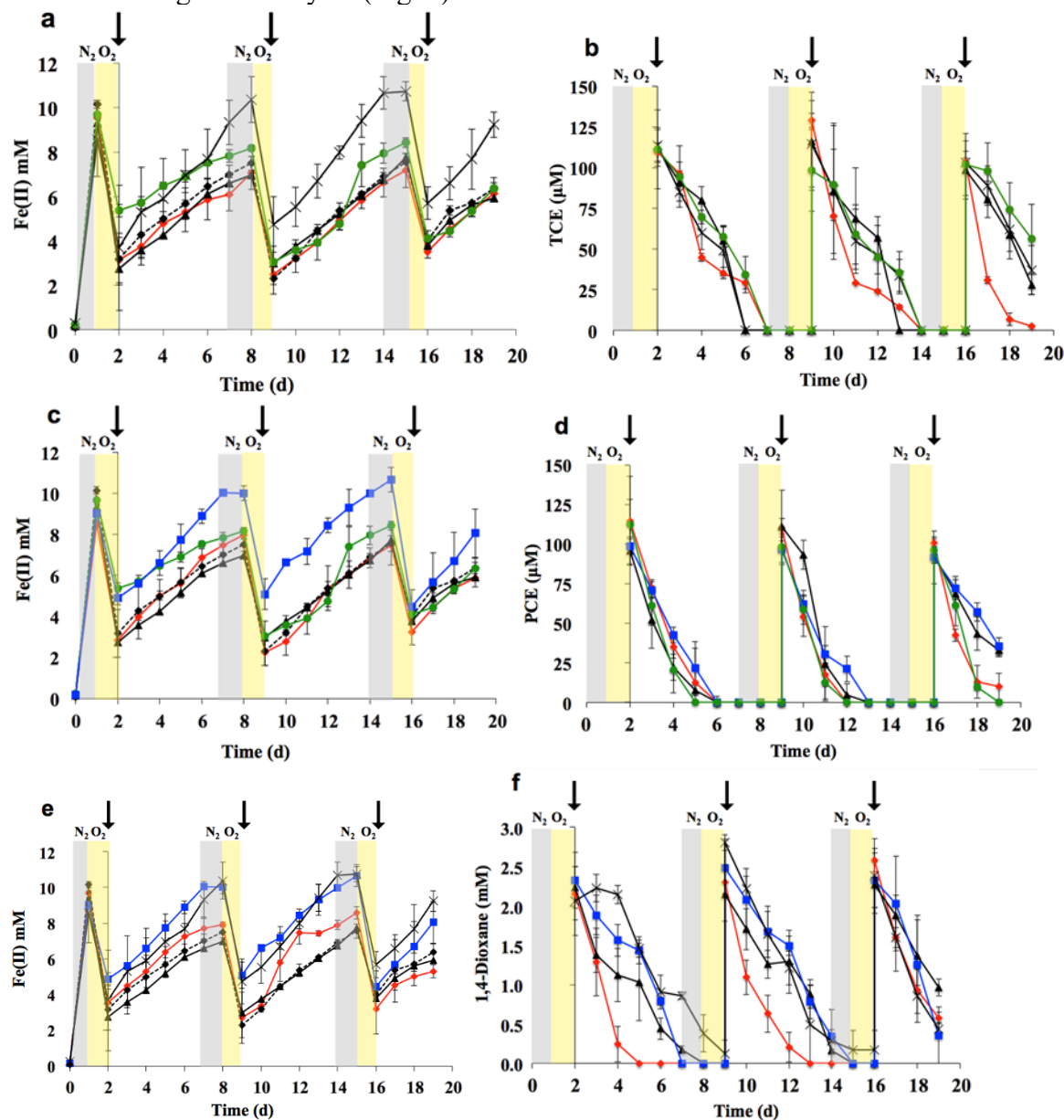


Figure 2. Concentration profiles during microbial Fenton degradation of TCE, PCE and 1,4-dioxane in fed batch liquid cultures of *S. oneidensis* amended with 10 mM Fe(III)-citrate, single, binary, and ternary

mixtures of TPD (100 μ M TCE, 100 μ M PCE and 2 mM 1,4-dioxane) and subjected to : Anaerobic, aerobic and TPD degradation phases: (a) Fe(II) (for TCE reactions); (b) TCE; (c) Fe(II) (for PCE reactions); (d) PCE; (e) Fe(II) (for 1,4-dioxane reactions); (f) 1,4-dioxane; solid red ($\blacklozenge$), single contaminant only; solid black (x), TCE + 1,4-dioxane; solid green ($\bullet$), TCE + PCE; solid blue ($\blacksquare$), PCE + 1,4-dioxane; solid black ($\blacktriangle$), TCE + PCE + 1,4-dioxane; dashed black ($\blacklozenge$), no contaminant control. Grey shaded areas correspond to Fe(II)-generating phase, yellow shaded areas correspond to H₂O₂-generating phase, and unshaded areas correspond to contaminant degradation phase. Arrows indicate time of addition (day 2) and respiking (day 9 & 16) of contaminants.

PCE Degradation in the Presence and Absence of TCE and 1,4-Dioxane. Nearly identical patterns of microbial (*S. oneidensis*-catalyzed) Fe(III) reduction and chemical (O₂-catalyzed) Fe(II) oxidation were observed in analogous PCE, TCE-PCE (TP), PCE-1,4-dioxane (PD), and TCE-PCE-1,4-dioxane (TPD) comingled contaminant degradation experiments (Figs. 2c). HO[•] radical production was initiated by the *S. oneidensis*-driven Fenton reaction. At the 2-d time point, the P, PT, PD, and TPD mixtures were added to initiate the first contaminant degradation phase. P was degraded as sole contaminant at nearly identical rates in the three successive contaminant degradation phases. P concentrations remained constant at 100 μ M in parallel control incubations lacking Fe(III) or *S. oneidensis* cells, but including three identical cycles of successive anaerobic (Fe(II)-generating), aerobic (H₂O₂-generating), and contaminant (P, PT, PD, and TPD) degradation phases. Identical patterns of microbial (*S. oneidensis*-catalyzed) Fe(III) reduction and chemical (O₂-catalyzed) Fe(II) oxidation were observed in parallel control incubations with contaminants omitted, which indicated that rates of microbially-catalyzed Fe(III) reduction and O₂-catalyzed Fe(II) oxidation were not affected by the presence of the P, PT, PD, or TPD mixtures.

1,4-Dioxane degradation in the presence and absence of TCE and PCE. Nearly identical patterns of microbial (*S. oneidensis*-catalyzed) Fe(III) reduction and chemical (O₂-catalyzed Fe(II) oxidation were observed in analogous D, DT, DP, and TPD contaminant degradation experiments (Fig. 2e). D was degraded at nearly identical rates as sole contaminant in the three successive contaminant degradation phases (2-7, 9-14, and 16-19 d time periods, Fig. 2e). D concentrations remained constant at 100 μ M in parallel control incubations lacking Fe(III) or *S. oneidensis* cells, but including three identical cycles of successive anaerobic (Fe(II)-generating), aerobic (H₂O₂-generating), and contaminant (D, DT, DP, and TPD) degradation phases. Identical patterns of microbial Fe(III) reduction and chemical Fe(II) oxidation were observed in parallel control incubations with contaminants omitted (Fig. 1e), which indicated that rates were not affected by the presence of the D, DT, DP, or TPD mixtures.

Similar to previous results reported with purely chemical Fenton reactions, results of the present study indicate that contaminant degradation rates in the microbially-driven Fenton reaction depend on the $k_{HO^{\bullet}}$ of competing contaminants, the initial contaminant concentration, and the number of double bonds in the contaminant molecular structure. In binary and ternary mixtures, contaminants with greater $k_{HO^{\bullet}}$ suppress the degradation of other contaminants with lower $k_{HO^{\bullet}}$ and the contaminant degradation phase must be extended for longer time periods to degrade contaminants below detection limits. The microbially-driven Fenton reaction may thus be applied as an effective *ex situ* platform for simultaneous degradation of at least three (and potentially more) co-mingled contaminants at source zone levels.

Optimizing the Microbially-Driven Fenton Reaction in Flow-Through Column Reactors. The FTCs loaded with ferrihydrite-coated sand and *S. oneidensis* were exposed to the contaminant 1,4-dioxane for about six weeks under fully aerated conditions to determine whether the generation of ROS in these conditions was able to promote the degradation of 1,4-dioxane. Two columns

were continuously injected either with aerated or degassed medium while 1,4-dioxane was added and removed twice from the input solution to simultaneously determine the effect of 1,4-dioxane on ROS production. Breakthrough curve of 1,4-dioxane revealed that a small fraction of 1,4-dioxane injected into the FTCs was removed from the input solutions, likely via reaction with ROS species as the fraction removed was much higher in the fully aerated flow-through column compared to the degassed and abiotic control columns (Fig. 3a). These findings provide strong evidence for the microbial-driven Fenton reaction in flow-through systems, although the reaction may not be as efficient as originally achieved in batch reactors. The experimental conditions were then varied to optimize the microbially-driven Fenton process. First, net rates of Fe(II), H₂O₂, and acetate production at the output of the FTCs indicated that the concentration of inorganic phosphate in the input medium had to be raised to at least 50 μ M to facilitate growth of *S. oneidensis*. Moreover, net rates of Fe(II) and H₂O₂ production decreased significantly when lactate concentration in the inlet medium was decreased from 5 to 1 mM. These findings suggest that a constant supply of 5 mM was enough to sustain microbial growth and that acetate consumption was enhanced in aerobic conditions at high lactate concentration, likely as a result of the significant increase in cell concentrations. Finally, the flow rate of the input solution into the FTCs was changed to examine its impact on microbially-driven Fenton reaction, and the net rate of both dissolved Fe(II) and H₂O₂ production increased proportionally to the flow rate.

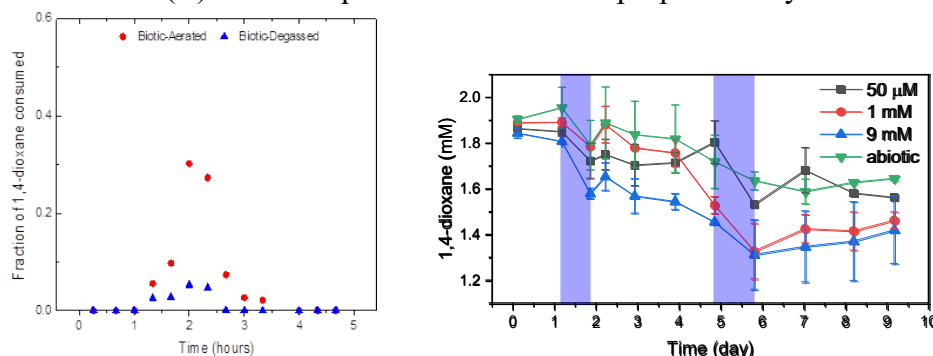


Figure 3. a. Breakthrough curve of 1,4-dioxane in biotic aerated and biotic degassed FTCs compared to the abiotic control. **b.** 1,4-dioxane degradation by *S. oneidensis* as a function of time during redox cycles in M1 medium amended with different orthophosphate concentrations (black squares, 50 μ M; red circles, 1 mM; blue triangles, 9 mM) relative to an abiotic control (green triangles). White areas correspond to the sealed periods, while blue shaded areas correspond to aerated periods.

Role of Orthophosphate and organic ligands in the Microbially-Driven Fenton Reaction. Batch reactor experiments demonstrated that higher orthophosphate concentrations are preferred to enhance bacterial growth in the reactors and that most of this growth appears to occur during the aerated phase of the incubations. In addition, high orthophosphate concentrations are preferred to promote the microbially-driven Fenton reaction, as low orthophosphate concentrations promote production of ferryl-ion intermediates that may decrease the production of hydroxyl radicals. In turn, high orthophosphate concentrations promote precipitation of iron phosphate minerals as secondary mineral products that may limit the recycling of Fe(III) oxyhydroxides. Thus, orthophosphate concentrations that are sufficiently high to promote growth and generate hydroxyl radicals are required to be able to sustain the microbially-driven Fenton reaction but not enough to exceed the solubility product of vivianite and other iron phosphate minerals which may scavenge iron and decrease the sustainability of the reaction. Secondary mineralization to goethite was evident in the low orthophosphate incubations and did not appear to prevent the microbially-driven Fenton reaction. Batch reactor experiments also demonstrated that 1,4-dioxane degradation by the

microbially-driven Fenton reaction may be less efficient with ferrihydrite than ferric citrate as terminal electron acceptor, even though recycling of Fe(III) during the first oxidative cycle in the ferric citrate incubations resulted in the production of an iron oxyhydroxide phase with the same reactivity as in the ferrihydrite incubations.

Application of the Microbially-Driven Fenton Reaction for Degradation of the Perfluoroalkyl (PFAS) Substances Perfluorooctanoic Acid (PFOA). After four cycles of the microbially driven Fenton reaction, endpoint samples from each bioreactor were analyzed for remaining PFOA. All bioreactors reduced and reoxidized Fe(III)-citrate at similar rates as expected in all cycles (Fig. 4a). PFOA extraction percent recoveries recovered almost all of the original 1 μM $\pm$ 20 nM (Fig. 5b), indicating the PFOA extraction method is robust enough to recover all PFOA. However, this also indicates no PFOA was degraded by the microbially-driven Fenton reaction. No bioreactors showed evidence of any PFOA degradation after MTBE extraction and LC-MS/MS analysis (no PFOA, 2%; experimental, 98%; abiotic, 101%; no Fe, 99%) when compared with the neat 1 μM control sample (Fig. 4b).

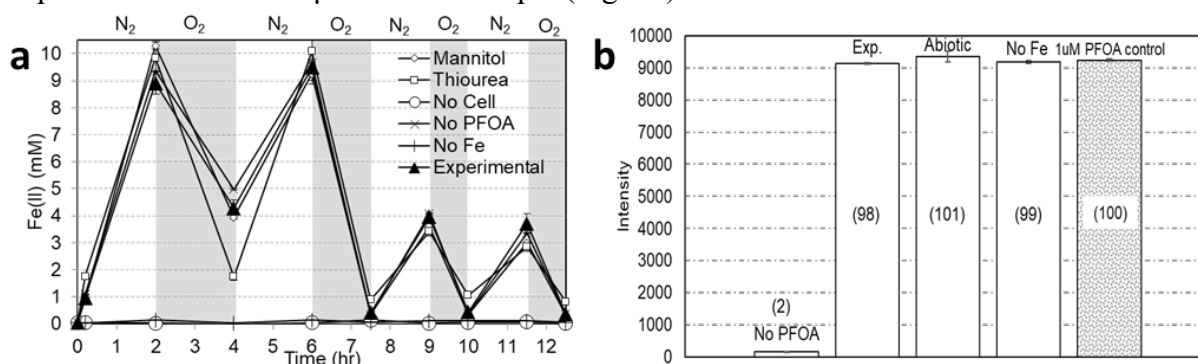


Figure 4. a. Four cycles of microbially driven Fenton bioreactors incubated with 1 μM PFOA. Error bars are present but may be hidden behind markers. **b.** Residual PFOA in bioreactors after MTBE extraction and LC-MS/MS quantitation (extraction percent recovery in parentheses, %).

We attempted several iterations of bioreactors, changing variables including cell density, cycling times, Fe(III)-citrate concentration, and cell growth medium, but no PFOA was degraded (representative data in Fig. 4). Recent literature asserts that PFOA is not degraded by the hydroxyl radical or superoxide, nor does the hydroxyl radical provide ancillary benefits to other radical attacks. Due to PFOA's recalcitrance to the microbially driven Fenton reaction and the hydroxyl radical, it was not prudent to pursue degradation of other PFAS via the microbially driven Fenton reaction.

Enhancement of the Microbially-Driven Fenton Reaction via Genetic Manipulation of Shewanella Genes Involved in Oxidative Stress Response. All single mutants had impaired exogenous H_2O_2 scavenging rates when compared with the wild-type under the conditions tested, with ΔpgpD again displaying the lowest scavenging rate (Fig 5a). ΔpgpD scavenged 20 μM exogenous H_2O_2 in 80 minutes, double the wild-type scavenging time, and displayed similarly depressed K_m and V_{max} when compared to wild-type (8.6 vs. 15.2 μM ; 0.5 vs 1.9 $\mu\text{M}/\text{min}$, respectively, Table 1 in full report). ΔkatGI again showed the second most affected scavenging rate (K_m 2.7 μM , V_{max} 0.5 $\mu\text{M}/\text{min}$) when compared with the wild type (Fig. 5a), but the K_m is likely skewed low due to the mutant switching kinetic strategies during the experiment.

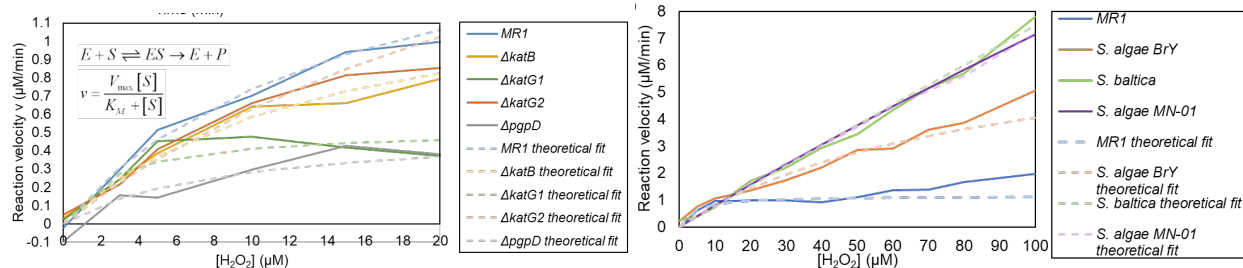


Figure 4. a. Michaelis-Menten saturation plots from whole-cell scavenging of exogenous H_2O_2 from 0-20 μM in minimal medium by wild-type *S. oneidensis* MR-1 and in-frame gene deletion mutants *S. oneidensis* $\Delta katB$, $\Delta katG1$, $\Delta katG2$, and $\Delta pgpD$. **b.** Michaelis-Menten saturation plots from whole-cell scavenging of exogenous H_2O_2 from 0-100 μM in minimal medium by wild-type *S. oneidensis* MR-1, *S. algae* BrY, *S. baltica*, and *S. algae* MN-01.

$\Delta katG1$ may experience a 20-minute acclimation period to H_2O_2 , during which it may upregulate or induce other genes that are able to compensate for KatG1 and rapidly scavenge any remaining H_2O_2 . Surprisingly, $\Delta katB$ and $\Delta katG2$ showed near-wild-type patterns of H_2O_2 scavenging (K_m 13.8, 13.2 μM ; V_{max} 1.4, 1.5 $\mu M/min$, respectively) (Table 1 in full report). In another study, these genes were the most upregulated under exogenous H_2O_2 stress. It is likely that loss of either KatB or KatG2 within a background of other functional scavenging enzymes is not enough to show a significant deleterious effect.

Following these results, double mutants were tested to determine whether there was more significant impairment of H_2O_2 scavenging rates upon concurrent loss of multiple scavenging proteins (Fig. 5.5 in full report). $\Delta katB\Delta katG1$ and $\Delta katB\Delta katG2$ scavenging rates were significantly impaired (K_m 6.4, 2.4 μM ; V_{max} 1.0, 0.4 $\mu M/min$, respectively) especially above 10 μM H_2O_2 . $\Delta katB\Delta pgpD$ and $\Delta katG1\Delta pgpD$ were also significantly impaired and took approximately twice as long as wild-type to clear exogenous H_2O_2 (K_m 3.1, 3.1 μM ; V_{max} 0.6, 0.3 $\mu M/min$, respectively). $\Delta katG2\Delta pgpD$ was mildly impaired when compared to wild-type scavenging time, but significantly impaired at scavenging kinetics (K_m 1.8 μM , V_{max} 0.4 $\mu M/min$). $\Delta katG1\Delta katG2$ scavenged H_2O_2 in a similar amount of time as wild-type (Fig. 5.5 in full report), but with different kinetics (K_m 4.9 μM , V_{max} 1.0 $\mu M/min$). Loss of only *katB* does not impact scavenging rates as much as loss of *katG1*, but when both *katB* and either *katG1* or *katG2* are absent from the genome, scavenging rates are severely affected. This suggests *S. oneidensis* likely utilize KatG1 or KatG2 in the absence of the main catalase KatB. Loss of PgpD in combination with loss of another scavenger also further impairs H_2O_2 scavenging rates, but not as severely. Under the conditions tested, all single mutant strains had similar rates of Fe(III) reduction (1mM/hr) and reoxidation (2.4mM/hr) (Fig. 5.6 in full report) with the wild-type strain. Clearly, mutants can still respire on Fe(III)-citrate at wild-type rates and this ability was not affected by lack of catalase or peroxidase. Double mutants were not tested. Incubations with a contaminant remains to be tested to determine whether mutants with reduced rates of H_2O_2 scavenging have enhanced 1,4-dioxane degradation rates.

Along similar lines, we tested other *Shewanella* species *S. algae* BrY, *S. baltica*, and *S. algae* MN-01 for their H_2O_2 scavenging rates, at a higher concentration range than previously (0-100 μM) as a comparison (Fig. 4b). *S. oneidensis* MR-1 was by far the weakest H_2O_2 scavenger tested (K_m 3.8 μM , V_{max} 1.1 $\mu M/min$), and appears to reach saturation above 40 μM , where the scavenging profile changes from zero order between 0-40 μM to first order above 40 μM . *S. baltica* and *S. algae* MN-01 were the strongest scavengers tested (K_m 4195.2, 806.1 μM ; V_{max} 320.3, 64.6 $\mu M/min$, respectively) and likely were not saturated at conditions tested. Consequently, K_m and V_{max} reported here may be excessively high, and these experiments need to be repeated at a higher

concentration range to determine true K_m and V_{max} . *S. algae* BrY (K_m 62.9 μ M, V_{max} 4.1 μ M/min) placed in between *S. oneidensis* and the other two species for H_2O_2 scavenging rate. Comparison of *S. oneidensis* H_2O_2 scavenging with ROS mutants and other *Shewanella* species indicates *S. oneidensis* as the weakest H_2O_2 scavenger tested. Perhaps the ability of *S. oneidensis* to degrade contaminants is in part due to poor ROS scavenging causing excessive extracellular hydroxyl radicals.

Implications for Future Research and Benefits

In previous applications of conventional (purely abiotic) Fenton reactions, 1,4-dioxane was degraded at rates approximately 20-fold greater than the microbially-driven Fenton reaction designed in the present study. Such differences may be due to the high concentrations (15 mM) of exogenous H_2O_2 employed to drive the chemical Fenton reaction systems. However, after normalization on a per mg protein basis, the reaction rates observed in the present study are 4-to-5-fold greater than the enzymatic rates of 1,4-dioxane degradation by *Pseudonocardia* species. In the present study, *S. oneidensis* produced micromolar levels of H_2O_2 , presumably as a by-product of microbial aerobic respiration during the aerobic cycling periods. Addition of exogenous H_2O_2 was therefore not required to drive the microbially-driven Fenton reaction. The chemical Fenton reaction also requires re-reduction of Fe(III) produced during H_2O_2 -catalyzed Fe(II) oxidation reactions. Fe(III) re-reduction processes such as those catalyzed by UV irradiation are possible during ex situ 1,4-dioxane degradation processes, yet UV light penetration represents a formidable obstacle for in situ 1,4-dioxane remediation technologies.

In the microbially-driven Fenton reaction designed in the present studies, *S. oneidensis* respiratory processes catalyze both H_2O_2 production and Fe(III) re-reduction. Since microbial Fe(III) reduction has been detected in a variety of aquatic environments, including contaminated subsurface aquifers, the microbially-driven Fenton reaction may be induced by exposing Fe(III)-reducing facultative anaerobes in Fe(III)-containing contaminated environments to alternating aerobic and anaerobic conditions. Alternately, the microbially-driven Fenton reaction may be stimulated by injecting soluble Fe(III), nanoparticulate zero-valent iron or electrolytic systems in the flow path of 1,4-dioxane-contaminated subsurface aquifers and exposing endogenous (or injected) Fe(III)-reducing bacteria to alternating aerobic and anaerobic conditions. Microbially-driven Fenton reaction may also be initiated through bioaugmentation at plume fringes where heterogeneous aerobic/anaerobic redox zonation may occur. In principal, targets for *ex situ* and *in situ* degradation by the microbially-driven Fenton reaction developed in the present study include multiple combinations of environmental contaminants susceptible to attack by Fenton reaction-generated $HO^\bullet$ radicals, including co-mingled plumes of 1,4-dioxane, PCE, and TCE. Unfortunately, PFOA is recalcitrant to the $HO^\bullet$ radical.

Flow through column and batch reactor demonstrated that iron oxyhydroxides could be used as terminal electron acceptor by *Shewanella oneidensis* MR-1 to produce Fe(II) that could be eventually exploited in the microbially-driven Fenton reaction to degrade organic contaminants. Our findings suggest either that growth rates in FTCs have to be optimized by altering nutrient (i.e., orthophosphate) concentrations or that Fe(III) oxyhydroxides are not as efficient as soluble Fe(III) substrates in promoting the microbially-driven Fenton reaction. The potential issues with medium and iron oxides were investigated in batch reactor incubations using redox oscillations to be able to promote the microbially-driven Fenton reaction with *S. oneidensis*. As organic ligands are known to affect the Fenton reaction, it is likely that stabilization of Fe(II) by ligands in solution as well as participation of the organic ligand in dark ROS production affects the generation of

hydroxyl radicals that are involved in organic contaminant degradation. The pandemic prevented to test these hypotheses more thoroughly. Future studies will compare incubations with and without citrate and other naturally occurring organic ligands (i.e. humics) to determine whether the presence of organic ligands may help improve the efficiency of the microbially-driven Fenton degradation of organic contaminants in flow-through column experiments. These findings will ultimately indicate whether the microbially-driven Fenton reaction occurs naturally in subsurface environments, potentially contributing to natural attenuation processes. In addition, the results from the flow-through column experiments conducted in this study demonstrate that the microbially-driven Fenton reaction is sustainable without high frequency redox oscillations, thus allowing us in future studies to transfer this technology to the pump-and-treat of contaminated subsurface waters.

S. oneidensis are well-known for their ability to respire on an extensive range of terminal electron acceptors, which has applications for bioremediation of heavy metal/radionuclide contaminated soil and groundwater. While the responsible anaerobic respiratory networks have been elaborated in detail, comparatively little has been studied about how *S. oneidensis* respond to oxidative stress, which is especially relevant during the aerobic cycles of the microbially driven Fenton reaction. *S. oneidensis* single and double mutants lacking ROS scavenging genes all displayed impaired H₂O₂ scavenging abilities, yet retained wild-type Fe(III)-reduction rates. All mutants need to be tested in a microbially driven Fenton bioreactor with 1,4-dioxane. Furthermore, comparison of *S. oneidensis* H₂O₂ scavenging with other *Shewanella* species demonstrates *S. oneidensis* as the weakest H₂O₂ scavenger tested. Perhaps the ability of *S. oneidensis* to degrade contaminants is due to such poor scavenging; if excessive H₂O₂ remains in the environment during the contaminant-degrading phase of the microbially driven Fenton reaction, there may be more availability for the OH• to attack and degrade contaminants. The genomes of the *Shewanella* species tested have similar arsenals of ROS scavenging genes, yet dramatically different H₂O₂ scavenging rates. Additional Fe(III)-reducing *Shewanella* species should be tested for contaminant degradation ability and rates. If more is understood about how *S. oneidensis* protect themselves from oxidative stress, it may be possible to enhance contaminant degradation rates.

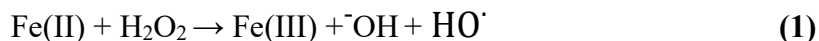
FULL REPORT

1. Microbially Driven Fenton Reaction for Degradation of the Widespread Environmental Contaminant 1,4-Dioxane.

1A. Objective. The main objectives of the present study were to i) design a microbially-driven Fenton reaction that autocatalytically generated HO[•] radicals and degraded 1,4-dioxane at circumneutral pH without the need for continual addition of exogenous H₂O₂ or UV irradiation to regenerate Fe(II), ii) optimize the 1,4-dioxane degradation rates by varying the duration and frequency of the aerobic and anaerobic incubation periods, and iii) determine the pathway for 1,4-dioxane degradation by identifying the transient intermediates produced during the microbially-driven 1,4-dioxane degradation process.

1B. Background. The carcinogenic cyclic ether compound 1,4-dioxane is detected in a variety of contaminated surface waters and groundwaters (1-5). 1,4-dioxane has been employed as a stabilizing agent for chlorinated solvents in the textile and paper industries (6,7) and as a byproduct of surfactant and polyethylene terephthalate plastic manufacturing processes (8-10). 1,4-dioxane is completely miscible in water, semi-volatile, and is thus highly mobile in water or aqueous environments. Current 1,4-dioxane remediation technologies such as carbon absorption, air stripping, and distillation are limited by problems associated with 1,4-dioxane solubility, boiling point, and vapor pressure, respectively (11). Alternate methods such as photo-remediation by UV light, ozone destruction in the presence of hydrogen peroxide (H₂O₂), and ultrasonic destruction are not cost-effective remediation strategies (12, 13).

Bioremediation is a promising alternative method for in situ remediation of 1,4-dioxane-contaminated waters. 1,4-dioxane may be degraded microbially via metabolic or cometabolic reactions. Microbial degradation is carried out aerobically by mixed microbial communities in industrial sludge (14, 15) and 1,4-dioxane-degrading bacteria have been isolated (16-19). Only a limited number of studies have examined microbial degradation of 1,4-dioxane under anaerobic conditions, and the degradation rates under nitrate-, iron-, and sulfate-reducing conditions are exceedingly slow (20). Ex situ treatment of 1,4-dioxane-contaminated ground water involves pumping and treatment via advanced oxidation processes (AOPs) (21-24). In the Fenton reaction (Eqn. 1), H₂O₂ reacts with ferrous iron (Fe(II)) to produce ferric iron (Fe(III)), hydroxyl ion (OH⁻), and hydroxyl radical):



Due to their high oxidation potential, Fenton reaction-generated HO[•] radicals oxidatively degrade a wide variety of hazardous organic compounds, including landfill leachates (25), chlorinated aliphatics and aromatics (26), dry-cleaning solvents (27), pharmaceuticals (28), pentachlorophenol (PCP) (29, 30), tetrachloroethene (PCE) (23), trichloroethene (TCE) (31) and 1,1,2-trichloroethane (TCA) (32). Fenton reaction-driven AOPs have also recently attracted attention as an alternative means for degrading 1,4-dioxane (33-36). Fenton reaction-driven AOPs are expensive, however, since the Fenton reagents Fe(II) and H₂O₂ must be continuously supplied to drive the chemical degradation reaction. UV irradiation is often employed to induce Fe(III) reduction and photolytic radical production in photo-Fenton systems. The UV irradiation systems, however, are limited by UV light penetration (37), and H₂O₂ must still be continuously supplied to drive the Fenton degradation reaction.

Microbially-driven Fenton reactions based on production of H_2O_2 via microbial O_2 respiration and Fe(II) via microbial Fe(III) reduction alleviate the need for continual addition of H_2O_2 and Fe(II) that drive the chemical Fenton reaction (36, 38, 39). The microbially-driven Fenton reaction designed in the present study was based on the original observation that the Fe(III) -reducing facultative anaerobe *Shewanella oneidensis* liquefied the agar support directly beneath colonies grown aerobically on solid growth medium supplemented with Fe(III) (40). Under such conditions, high microbial O_2 consumption rates directly beneath the colony effectively lower the O_2 concentrations to levels that permit simultaneous microbial Fe(III) reduction (41), concomitant production of the Fenton substrates H_2O_2 and Fe(II) , and agar liquefaction via the resulting $\text{HO}^\bullet$ radicals (Fig. 1). The agar liquefaction phenotype was subsequently employed as the basis of a genetic screening technique for identification of Fe(III) reduction-deficient mutant strains and the corresponding genes required for microbial Fe(III) reduction (40, 42).

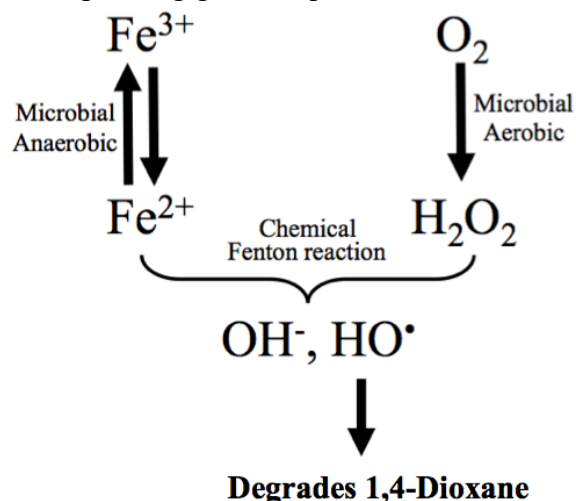


Figure 1. Overall strategy for generation of $\text{HO}^\bullet$ radicals by the *S. oneidensis*-driven Fenton reaction. Fe(II) produced during anaerobic phases interacts chemically via the Fenton reaction with H_2O_2 produced during aerobic phases to yield $\text{HO}^\bullet$ radicals that oxidatively degrade 1,4-dioxane.

1C. Materials and Methods. Design of a Microbially Driven Fenton Reaction for 1,4-Dioxane Degradation. The toxicity of 1,4-dioxane to *S. oneidensis* was tested by growing batch cell cultures in the presence of potential source zone levels (10 mM) of 1,4-dioxane in LS medium under aerobic conditions for 48 h. Culture samples were withdrawn periodically during the toxicity tests and the number of colony forming units (CFUs) were measured to monitor cell viability. The experimental conditions for the microbially-driven Fenton degradation of 1,4-dioxane (designated DD conditions in the remainder of the present study) consisted of *S. oneidensis* batch cultures amended with Fe(III) and 1,4-dioxane and exposed to different aerobic/anaerobic cycling periods. *S. oneidensis* was grown aerobically in LB on a rotary shaker (200 rpm, 30 °C) to early stationary phase ($\text{OD}_{600} = 1.5$), harvested by centrifugation at 6000 x g, washed and resuspended in LS medium to a final cell density of 1×10^9 cells per ml. Anaerobic stock solutions of Fe(III) citrate and 1,4-dioxane were added to final concentrations of 10 mM each. The cell culture was allowed to reduce Fe(III) citrate for pre-selected time periods (45 min, 1.5 h, 3 h, and 6 h) under anaerobic conditions maintained by continuously sparging with hydrated high-purity nitrogen. Reactor temperature (25°C) and pH (7.0) were held constant in all experiments. Aerobic conditions were initiated by sparging the culture with hydrated compressed air for pre-selected time periods (45 min, 1.5 h, 3 h, and 6 h). Cell density was monitored by determining colony forming units on LB agar plates incubated at 30 °C for 72 h.

Chemical Analysis of 1,4-Dioxane, Lactate, and Transient Degradation Products. Samples were withdrawn and centrifuged at 6000 x g for 10 min. 1,4-dioxane and ethylene glycol diformate were analyzed via liquid chromatography (LC) using a ZORBAX SB-C18 column with 20% aqueous acetonitrile as the mobile phase and a constant flow rate of 1.0 ml/min (18). Chromatograms were generated at 190 nm for 1,4-dioxane at a retention time of 2.4 min and at 210 nm for ethylene glycol diformate at a retention time of 3.1 min²⁴. Lactate, acetate, formate, glyoxylate, glycolate and oxalate were analyzed via an ion chromatograph (Dionex, DX-300 Series) equipped with a Dionex IonPac® ICE-AS6 chromatography column and AMMS® ICE 300 suppressor. The Dionex DX-300 uses a CDM II detector with suppressed conductivity detection. Anion analysis was performed with 0.4 mM heptafluorobutyric acid as eluent and 5 mM tetrabutylammonium hydroxide as regenerant. Chromatograms were generated for lactate, acetate, oxalate, formate, glyoxylate, and glycolate at retention times of 10.0, 13.3, 4.3, 9.3, 6.5, and 9.0 min, respectively. Calibration curves were generated from standards to determine the concentrations of each compound.

Inhibition of the Microbially-Driven Fenton Reaction. A series of five control experiments were carried out to confirm that 1,4-dioxane was degraded by HO[•] radicals generated by the *S. oneidensis*-driven Fenton reaction. All five control experiments were carried out under DD conditions, with the following noted changes: In the first set of control experiments, 1,4-dioxane degradation was monitored under identical alternating aerobic/anaerobic periods with 15 mM NO₃⁻ replacing Fe(III) as electron acceptor. In the second set of control experiments, the HO[•] radical scavenging compounds mannitol (120 mM) and thiourea (40 mM) were added to reactors carrying out an otherwise identical 1,4-dioxane degradation process (30). The toxicity of mannitol (120 mM) and thiourea (40 mM) to *S. oneidensis* was tested by growing batch cell cultures in the presence of the HO[•] radical scavenging compounds in LS medium under aerobic conditions for 48 h. In the third set of control experiments, 1,4-dioxane degradation was monitored under DD conditions with either Fe(III) citrate, 1,4-dioxane, or cells (abiotic control) omitted. In the fourth set of control experiments, 1,4-dioxane concentration was monitored in abiotic sealed anaerobic bottles to examine the effects of hydrated compressed air and nitrogen gas flow on volatilization of 1,4-dioxane during the aerobic and anaerobic phases, respectively. In the fifth set of control experiments, 1,4-dioxane concentrations were monitored under strictly aerobic conditions for 74 h. Analytical methods are fully described in (59).

1D. Results and Discussion. In the present study, a microbially-driven Fenton reaction was designed to degrade the widespread environmental contaminant 1,4-dioxane. The microbially-driven Fenton reaction autocatalytically generated HO[•] radicals that degraded 1,4-dioxane at circumneutral pH without the need for continual addition of exogenous H₂O₂ or UV irradiation to regenerate Fe(II) as Fenton reagents. The 1,4-dioxane degradation process was driven by pure cultures of the Fe(III)-reducing facultative anaerobe *S. oneidensis* provided with lactate as carbon and energy source, Fe(III) as electron acceptor, and exposed to alternating aerobic and anaerobic conditions (Fig. 3).

H₂O₂ produced during the aerobic period was most likely the byproduct of microbial aerobic respiration since H₂O₂ was not detected during abiotic O₂-catalyzed Fe(II) oxidation experiments. Alternate aerobic/anaerobic cycling periods of 3 h and 6 h resulted in the maximum rate and extent of H₂O₂ production, both of which were nearly identical to the rate and extent of H₂O₂ production under strictly aerobic conditions. H₂O₂ production with 45 min and 1.5 h cycling periods resulted in lower rates and extents of H₂O₂ production (Fig. 2).

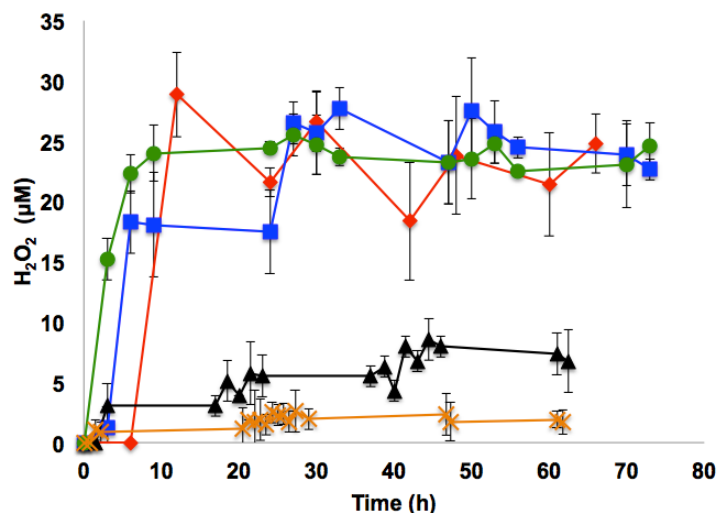


Figure 2. H₂O₂ production by *S. oneidensis* held under strictly aerobic conditions or with aerobic/ anaerobic cycling periods of 45 min, 1.5 h, 3 h and 6 h: red $\blacklozenge$, 6 h cycling; blue $\blacksquare$, 3 h cycling; black $\blacktriangle$, 1.5 h cycling; green $\bullet$, strict aerobic; orange $\times$, 45 min cycling.

During the transition from anaerobic-to-aerobic conditions, *S. oneidensis* batch cultures displayed lag times in H₂O₂ production. H₂O₂ production rates were maximal during the 3 h and 6 h aerobic/anaerobic cycling periods, but 3-to-10-fold lower during the 45 min and 1.5 h cycling periods (Fig. 2). In addition, the 3 h aerobic/anaerobic cycling period was sufficient to achieve the maximum rate and extent of H₂O₂ production, which was reflected in the results of the 1,4-dioxane degradation experiments described below.

H₂O₂ production by *S. oneidensis*. In iron-free LS medium, *S. oneidensis* batch cultures produced approximately 24.5 μM H₂O₂ (presumably as a by-product of microbial aerobic respiration) under either strictly aerobic conditions for 74 h or with alternating aerobic/anaerobic periods of 3 h and 6 h (Fig. 2). Under aerobic/anaerobic cycling periods of 45 min and 1.5 h, *S. oneidensis* produced 2.3 μM and 6.7 μM H₂O₂, respectively (Fig. 2). During the first aerobic phase, H₂O₂ production rates under strictly aerobic conditions or with alternating aerobic/anaerobic periods of 3 h and 6 h were similar (4.6-5.6 μM). However, H₂O₂ production rates with alternating aerobic/anaerobic cycling periods of 45 min and 1.5 h were 4-fold lower (1.2 μM) compared to the strictly aerobic and 3 h and 6 h aerobic/anaerobic cycling conditions (Fig. 2). During the second aerobic phase, H₂O₂ production rates with the 3 h and 6 h aerobic/anaerobic cycling periods decreased 5-fold and were similar to H₂O₂ production rates with aerobic/anaerobic cycling periods of 45 min and 1.5 h (0.8-1.3 μM) (Fig. 2). During the third aerobic phase, H₂O₂ production rates with aerobic/anaerobic cycling periods of 45 min, 1.5 h, 3 h, and 6 h were similar (0.4-0.9 μM) (Fig. 2). H₂O₂ was not detected during abiotic Fe(II) oxidation experiments carried out under strictly aerobic conditions for 74 h (data not shown).

1,4-Dioxane Degradation by the Microbially-Driven Fenton Reaction. To initiate HO $\cdot$ radical production by the *S. oneidensis*-driven Fenton reaction, Fe(III)-containing *S. oneidensis* cultures were exposed to alternating aerobic/anaerobic periods of 45 min, 1.5 h, 3 h, and 6 h (Fig. 3 and Supplementary Figs. S3, S4, and S5 in (59)). During the initial 3 h anaerobic and ensuing 3 h aerobic periods, 1,4-dioxane concentrations decreased 2.4 mM in the incubation under DD conditions (Fig. 3b). However, 1,4-dioxane concentrations also decreased 1.5 mM in control DD

incubations lacking Fe(III) or *S. oneidensis* cells (Fig. 3b). The decrease in 1,4-dioxane concentrations in the control incubations was attributed to 1,4-dioxane volatilization during hydrated compressed air and nitrogen inputs. To address this possibility, a series of additional control experiments were carried out in which 1,4-dioxane concentrations were monitored under DD conditions with *S. oneidensis* cells omitted and subjected to a series of five 3 h anaerobic/aerobic cycling periods for 74 h (Fig. 3b). 1,4-dioxane concentrations decreased 46% (from 12.0 to 6.5 mM) during the first 9 h of the 74 h incubations. On the other hand, in control incubations held under strict anaerobic conditions for 74 h without hydrated compressed air or nitrogen inputs, 1,4-dioxane concentrations remained constant at 10.0 mM (Fig. 3b). These results indicate that during the first 9 h of the 74 h control incubations (consisting of alternating 3 h aerobic/anaerobic cycling periods) 1,4-dioxane concentrations decreased approximately 46% (to 5.5 mM) due to 1,4-dioxane volatilization. In a similar manner, 1,4-dioxane volatilization in control reactions with alternating compressed air and nitrogen inputs during the 45 min, 1.5 h and 6 h aerobic/anaerobic cycling experiments resulted in decreases of approximately 35%, 37% and 50% of the initial 1,4-dioxane concentrations, respectively (Supplementary Figs. S4-b, S3-b and S5-b respectively in (59)).

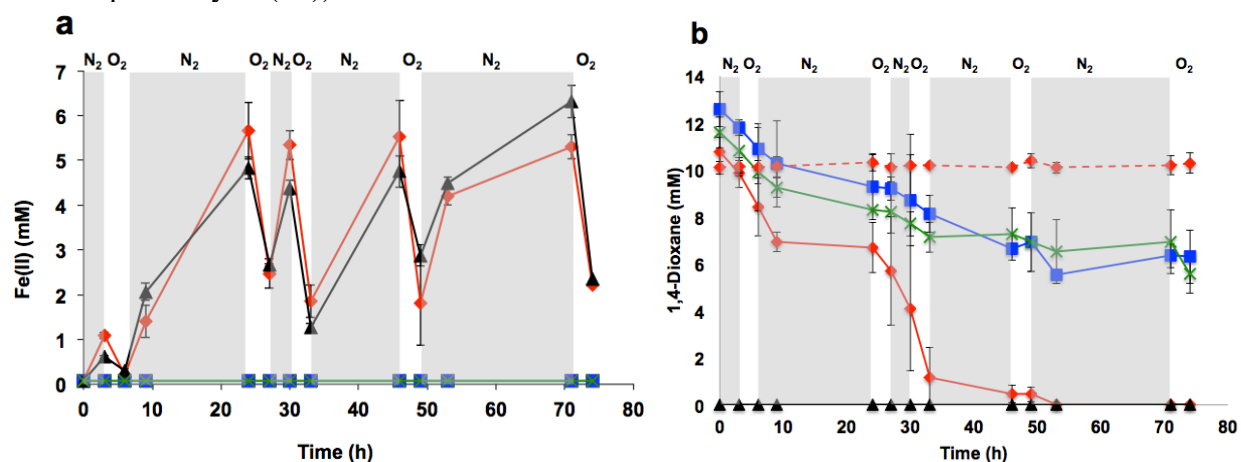


Figure 3. Fe(II) and 1,4-dioxane concentrations during microbially-driven Fenton degradation of 1,4-dioxane (10 mM initial concentration) with an aerobic/anaerobic cycling period of 3 h: (a) Fe(II); (b) 1,4-dioxane; red $\blacklozenge$, cells + 1,4-dioxane + Fe(III); blue $\blacksquare$, *S. oneidensis* cells omitted; black $\blacktriangle$, 1,4-dioxane omitted; green $\times$, Fe(III) omitted; dashed red $\blacklozenge$, No Gas control. Grey shaded areas correspond to anaerobic phases and unshaded areas correspond to aerobic phases. Incubations were carried out in two parallel yet identical cultures and error bars indicate range of error between incubations.

During the first 3 h anaerobic period under DD conditions, *S. oneidensis* reduced 10.0 mM Fe(III) to 1.1 mM Fe(II) (grey shaded area in Figs. 2a-b, 3a-c, 4a-b, 5a-c and Supplementary Figs. S2, S3a-f, S4a-f, S5a-f in (59)). At the 3-h mark, compressed nitrogen gas input was switched to compressed air, Fe(II) was oxidized for a 3 h incubation period (unshaded area in Figs. 2a-b, 3a-c, 4a-b, 5a-c and Supplementary Figs. S2, S3a-f, S4a-f, S5a-f in (59)), and Fe(II) levels dropped to below detection limits at the 6-h time point. During the ensuing 3 h anaerobic period, Fe(II) rebounded from below detection limits to 5.5 mM (microbial Fe(III) reduction rate of 0.3 mM/h). At the 9-h time point, 1,4-dioxane volatilization stopped (even though compressed nitrogen inputs were continued to ensure anaerobic conditions for the ensuing 18 h) and 1,4-dioxane concentrations remained constant at 7.0 mM (i.e., corresponding to the observed volatilization losses of 6.5 mM 1,4-dioxane during the initial 9 h period). After the ensuing 3 h aerobic period

(24-h time point), Fe(II) levels decreased from 5.5 to 2.6 mM (O_2 -catalyzed Fe(II) oxidation rate of 1.0 mM/h). After the next 3 h anaerobic period (27-h time point), the Fe(II) concentrations again rebounded to 5.5 mM (microbial Fe(III) reduction rate of 1.1 mM/h). During the 9 h time period between the 24-h and 33-h time points, 1,4-dioxane concentrations decreased rapidly to 1 mM (0.6 mM/hr). However, in control incubations carried out under DD conditions but lacking *S. oneidensis* cells or Fe(III), 1,4-dioxane concentrations remained constant at 7.0 mM and remained at that concentration throughout the remainder of the 74 h incubation. At the 49-h time point, the incubations under DD conditions were subjected to two additional alternating 3 h anaerobic/aerobic cycling periods that resulted in production of 6.3 mM Fe(II) (71-h time point; microbial Fe(III) reduction rate of 0.2 mM/h) followed by a decrease in Fe(II) concentrations to 2.2 mM (74-h time point; O_2 -catalyzed Fe(II) oxidation rate of 1.4 mM/h) (Fig 2a). 1,4-dioxane concentrations decreased to below detection limits at the 53-h time point and remained below detection limits (0.2 mM; Supplementary Table S3) throughout the remainder of the 74 h incubation.

Since 1,4-dioxane is a semi-volatile compound, approximately 46% of the decrease in 1,4-dioxane concentrations was attributed to volatilization during hydrated compressed gas inputs, while the remaining 54% decrease in 1,4-dioxane concentrations to below detection limits (0.2 mM; Supplementary Table S3) was due to degradation by $HO^\bullet$ radicals produced by the microbially-driven Fenton reaction (47). Approximately 46% of the initial 1,4-dioxane concentration was removed by volatilization during the hydrated gas inputs due to continual diffusion of 1,4-dioxane to the vapor phase until vapor-liquid equilibrium was attained at 9 h. 1,4-dioxane was not further volatilized since the compressed gases were saturated with water prior to injection (Fig. 3b and Supplementary Fig. S7 in (59)).

Similar patterns of microbial (*S. oneidensis*-catalyzed) Fe(III) reduction and chemical (O_2 -catalyzed) Fe(II) oxidation were observed in analogous experiments carried out with 45 min, 1.5 h, 3h, and 6 h aerobic/anaerobic cycling periods (Supplementary Figs. S4-A, S3-A and S5-A respectively in (59)). Compared to the 3 h aerobic/anaerobic cycling experiments, 1,4-dioxane degradation rates were 34% slower during the 1.5 h aerobic/anaerobic cycling experiment (Supplementary Fig. S3-b in (59)). 1,4-Dioxane was not degraded (i.e., depletion was only due to volatilization) during the 45 min aerobic/anaerobic cycling experiment (Supplementary Fig. S4-b in (59)). However, 1,4-dioxane degradation rates with 3 h and 6 h aerobic/anaerobic cycling periods were nearly identical (Fig. 3b and Supplementary Fig. S5-b (59)).

Cell viability analyses indicated that *S. oneidensis* cell densities decreased approximately 70% during the 3 h anaerobic periods, yet rebounded to initial cell densities (10^9 cells/ml) during subsequent 3 h aerobic periods (Supplementary Figs. S2, S3-f and S4-f (59)). To test for 1,4-dioxane toxicity, *S. oneidensis* was grown aerobically in LS growth medium supplemented with 10 mM 1,4-dioxane. Aerobic growth rates in the presence or absence of 1,4-dioxane were nearly identical, an indication that 10 mM 1,4-dioxane was not toxic to *S. oneidensis* (Supplementary Fig. S1 in (59)). Although cell densities decreased approximately 70% during the 3 h anaerobic incubation periods, lactate was consumed at similar rates (0.21 mM/h) during the 3 h aerobic and anaerobic cycling periods. Lactate was not depleted in abiotic control incubations carried out with otherwise identical aerobic and anaerobic cycling periods (Fig. 4a).

Inhibition of the Microbially-Driven 1,4-Dioxane Degradation Process. To determine if $HO^\bullet$ radical scavenging compounds inhibited the microbially-driven Fenton reaction, a series of control incubations were carried out under DD conditions in the presence of the $HO^\bullet$ radical scavenging compounds mannitol (120 mM) or thiourea (40 mM) (Fig. 5b). 1,4-dioxane

concentrations in all incubations decreased to 6.0 mM during the initial 9 h aerobic and anaerobic cycling periods (due to 1,4-dioxane volatility; as described above). 1,4-dioxane was degraded to below detection limits (0.2 mM; Supplementary Table S3) after 53 h of alternating 3 h aerobic/anaerobic cycling periods in the absence of mannitol or thiourea (Fig. 5b), while 1,4-dioxane remained constant at 6.0 mM in the presence of mannitol or thiourea. To test for toxicity effects of mannitol and thiourea, *S. oneidensis* was grown aerobically in LS growth medium supplemented with mannitol (120 mM) or thiourea (40 mM). Aerobic growth rates in the presence or absence of mannitol or thiourea were nearly identical, an indication that these compounds were not toxic to *S. oneidensis* (Supplementary Fig. S6 in (59)). The inability of the microbially-driven Fenton reaction to degrade 1,4-dioxane in the presence of HO[•] radical scavenging compounds mannitol or thiourea or in the absence of either *S. oneidensis* cells or Fe(III) implicated HO[•] radicals as the main driver of the 1,4-dioxane degradation process.

The requirement for microbial Fe(III) reduction was tested by replacing Fe(III) with NO₃⁻ and carrying out an otherwise identical set of DD experiments with NO₃⁻-containing *S. oneidensis* cultures subjected to alternating 3 h aerobic/anaerobic cycling periods. 1,4-dioxane was not degraded with NO₃⁻ as electron acceptor (Fig. 5b), nor was 1,4-dioxane degraded in the absence of Fe(III) or *S. oneidensis* cells (Fig. 3b). These results indicate that microbial Fe(III) reduction was required to drive the 1,4-dioxane degradation process. In all control incubations, lactate was consumed at similar rates (0.21 mM/h) under either strictly aerobic or anaerobic Fe(III)-reducing conditions. Lactate concentrations remained constant in 74 h abiotic control incubations held under strictly aerobic or anaerobic conditions (Fig. 6a). Apart from the 45% loss due to volatilization, 1,4-dioxane was not degraded under strictly aerobic conditions, which is identical to previously reported findings (Supplementary Fig. S7 in (59)) (30). The absence of 1,4-dioxane degradation under strictly aerobic or strictly anaerobic Fe(III)-reducing conditions indicates that 1,4-dioxane degradation was not due to aerobic or anaerobic enzymatic 1,4-dioxane degradation reactions catalyzed by *S. oneidensis* (2, 19).

Identification of Transient 1,4-Dioxane Degradation Products During the Microbially-Driven Fenton Reaction. The major transient 1,4-dioxane degradation products detected during conventional (purely abiotic) Fenton reactions include formate, glycolate, glyoxylate, ethylene glycol diformate, acetate, and oxalate, which are produced by HO[•] radical attack of 1,4-dioxane with the α -oxy radical as key intermediate (36). With LC/UV-DA and IC detection methods, only acetate and oxalate were detected as transient 1,4-dioxane degradation products during the microbially-driven Fenton reaction. In the first 3 h anaerobic period, 0.7 mM acetate (at a rate of 0.24 mM/h) was produced under DD conditions, while acetate concentrations remained below detection limits (40.38 μ M) in DD control incubations lacking *S. oneidensis* cells or Fe(III) or in the presence of HO[•] scavenging compounds (Fig. 5b).

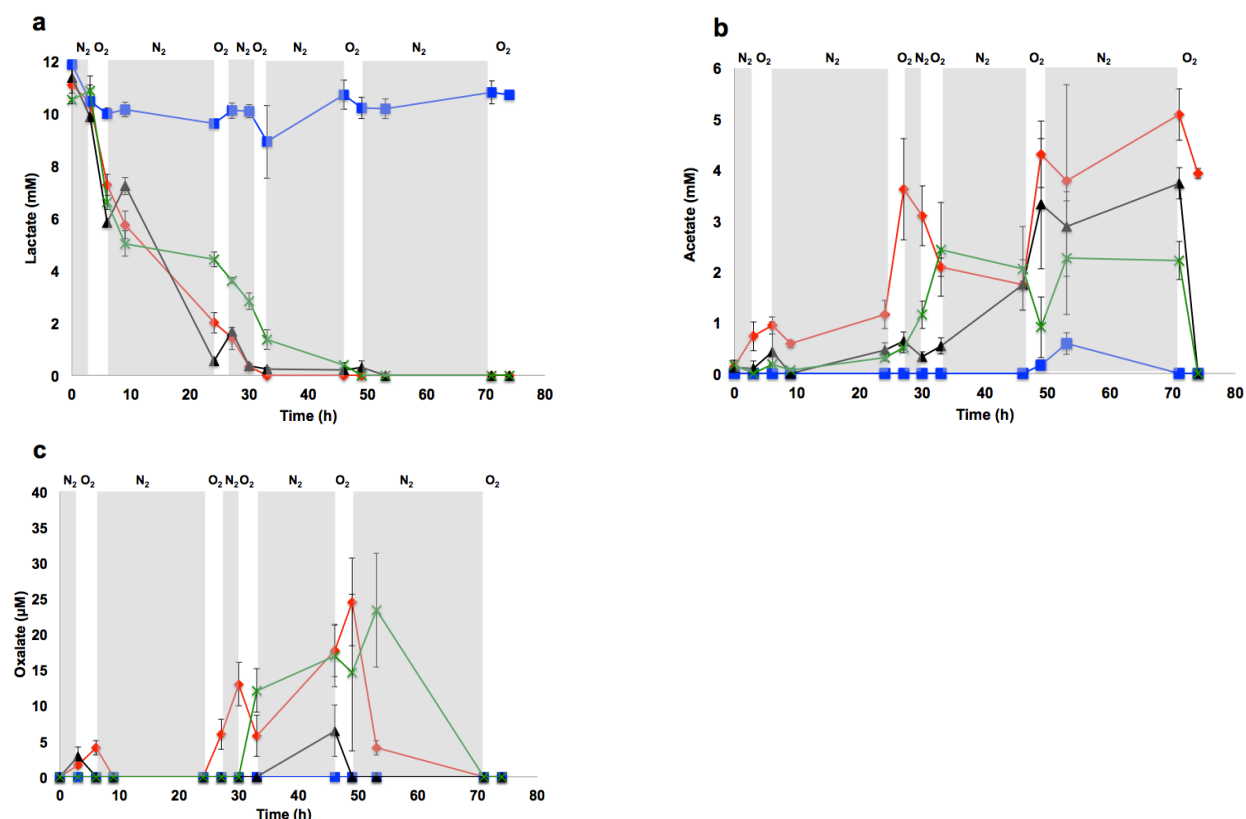


Figure 4. Lactate, acetate, and oxalate concentrations during microbially-driven Fenton degradation of 1,4-dioxane (10 mM) with an aerobic/anaerobic cycling period of 3 h: (a) lactate; (b) acetate; (c) oxalate; red $\blacklozenge$, cells + 1,4-dioxane + Fe(III); blue $\blacksquare$, *S. oneidensis* cells omitted; black $\blacktriangle$, 1,4-dioxane omitted; green $\times$, Fe(III) omitted. Grey shaded areas correspond to anaerobic phases and unshaded areas correspond to aerobic phases.

During the ensuing 3 h aerobic period of all biotic incubations, acetate concentrations increased at similar rates (0.08 mM/h) (Fig. 4b), presumably due to the microbially catalyzed aerobic oxidation of lactate. Acetate concentrations remained constant during the next 18 h anaerobic period. Upon introduction of compressed air at the 24-h time point under DD conditions, acetate concentrations increased rapidly at the rate of 0.81 mM/h (Fig. 4b). During this same time period, 1,4-dioxane concentrations decreased sharply (0.33 mM/h) as compared to decreases in 1,4-dioxane concentrations (0.07 mM/h) in DD control incubations lacking *S. oneidensis* cells or Fe(III) (Fig. 3b).

During the next set of aerobic/anaerobic cycling periods, acetate concentrations decreased sharply from 3.6 mM to 2.1 mM at the 33-h time point (0.25 mM/h). At the 49-h time point, acetate production rates (1.13 mM/h) under DD conditions increased approximately 45% compared to DD control incubations (0.80 mM/h) lacking *S. oneidensis* cells or Fe(III). Higher acetate production rates are most likely due to 1,4-dioxane (and not lactate) degradation since lactate had been completely depleted by the 46-h time point (Figs. 3b and 6b). Since lactate was also completely depleted in all reactions containing *S. oneidensis* cells, microbial oxidation of lactate to acetate most likely contributed to acetate production (Figs. 3a and 5a). Acetate concentrations in the incubations containing the HO $\cdot$ radical scavenging compounds mannitol or thiourea, however, remained constant at 1-2 mM throughout the 74 h incubation period (Fig. 7b).

In addition, the total acetate produced under DD conditions with 3 h aerobic/anaerobic cycling periods was 7.7 mM (approximately 2-fold greater than that detected in the control incubations (2.5-4.9 mM) (Table 1). The 2-to-3-fold greater amount of acetate produced in the presence of 1,4-dioxane was reflected in the 2-to-4-fold greater amount of 1,4-dioxane degraded in the microbially-driven Fenton reaction compared to DD control incubations lacking *S. oneidensis* cells or Fe(III) or in the presence of mannitol or thiourea (Table 1). Similar results were obtained in DD incubations with aerobic/anaerobic cycling frequencies of 1.5 h, 45 min, and 6 h (Supplementary Table S1).

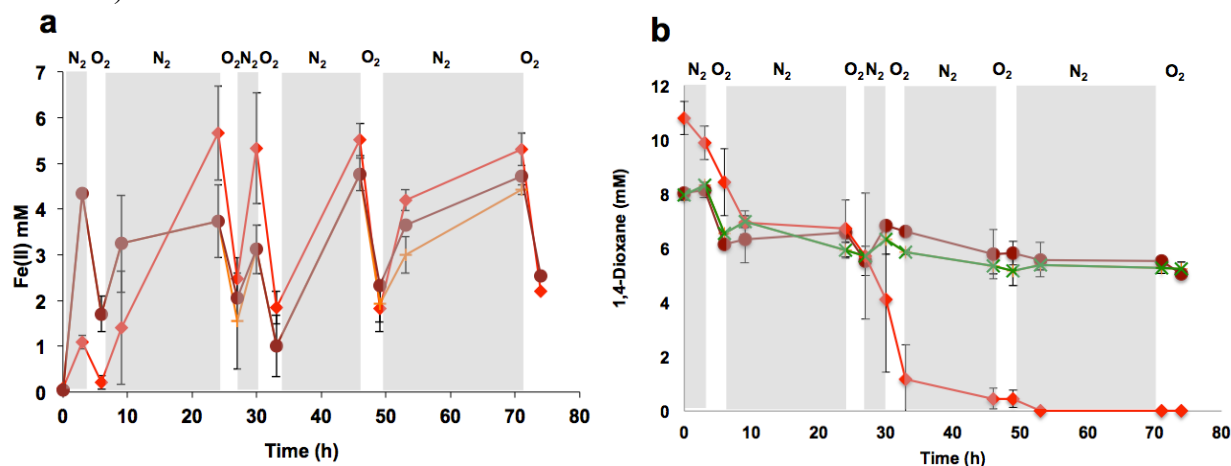


Figure 5. Fe(II) and 1,4-dioxane concentrations during the microbially-driven Fenton degradation of 1,4-dioxane (10 mM) with an aerobic/anaerobic cycling period of 3 h in the presence of inhibitors: (a) Fe(II); (b) 1,4-dioxane; red $\blacklozenge$, *S. oneidensis* cells + 1,4-dioxane + Fe(III); orange $+$, mannitol; brown $\bullet$, thiourea; green $\times$, nitrate. Grey shaded areas correspond to anaerobic phases and unshaded areas correspond to aerobic phases. Incubations were carried out in two parallel yet identical cultures and error bars indicate range of error between incubations.

	3 h cycling			
	Degraded (mM)		Produced (mM)	
	1,4-Dioxane	Lactate	Acetate	Oxalate
Cells + 1,4-dioxane+Fe(III)	10.8 $\pm$ 0.6	11.1 $\pm$ 0.6	7.7 $\pm$ 0.8	0.04 $\pm$ 0.002
Cells omitted	6.3 $\pm$ 0.4	1 $\pm$ 0.3	0	0
1,4-Dioxane omitted	0	11.4 $\pm$ 0.4	4.8 $\pm$ 0.4	0.01 $\pm$ 0.002
Fe(III) omitted	6 $\pm$ 0.2	10.5 $\pm$ 0.1	3.9 $\pm$ 0.4	0.03 $\pm$ 0.004
Mannitol	2.8 $\pm$ 0.5	9.3 $\pm$ 0.3	2.8 $\pm$ 0.3	0.01 $\pm$ 0.009
Thiourea	3 $\pm$ 0.01	9.5 $\pm$ 0.03	4.7 $\pm$ 0.6	0.01 $\pm$ 0.004
Nitrate	2.8 $\pm$ 2.4	9.5 $\pm$ 0.1	2.5 $\pm$ 0.3	0.01 $\pm$ 0.006

Table 1. Substrate degradation and transient intermediate production during the microbially-driven Fenton degradation of 1,4-dioxane. Incubations were carried in two parallel yet identical cultures and error bars indicate range of error between incubations.

Approximately 4 μ M oxalate was produced during the initial set of 3 h aerobic/anaerobic cycling periods under DD conditions, while oxalate was not detected in DD control incubations lacking *S. oneidensis* cells or Fe(III) (Figs. 3c and 6c). At the 24-h time point,

oxalate concentrations under DD conditions steadily increased to 13.0 μM at a rate of 2.2 $\mu\text{M}/\text{h}$. At the 33-h time point, strictly anaerobic conditions were held for the ensuing 18 h and oxalate concentrations increased to 17.7 μM at a rate of 0.9 $\mu\text{M}/\text{h}$ (2-fold greater than control incubations lacking *S. oneidensis* cells or Fe(III)). At the 46-h time point, oxalate concentrations increased further to 24.5 μM during the 3 h aerobic incubation period (Figs. 3c and 5c). Approximately 0.04 mM oxalate was produced under DD conditions (2-to-4-fold greater than control incubations lacking *S. oneidensis* cells or Fe(III), Table 1). Results similar to those of the 3 h aerobic/anaerobic cycling period experiments were obtained with aerobic/anaerobic cycling periods of 45 min, 1.5 h, and 6 h (Supplementary Figs. S3e, S4e, S5e and Supplementary Table S1 in (59)).

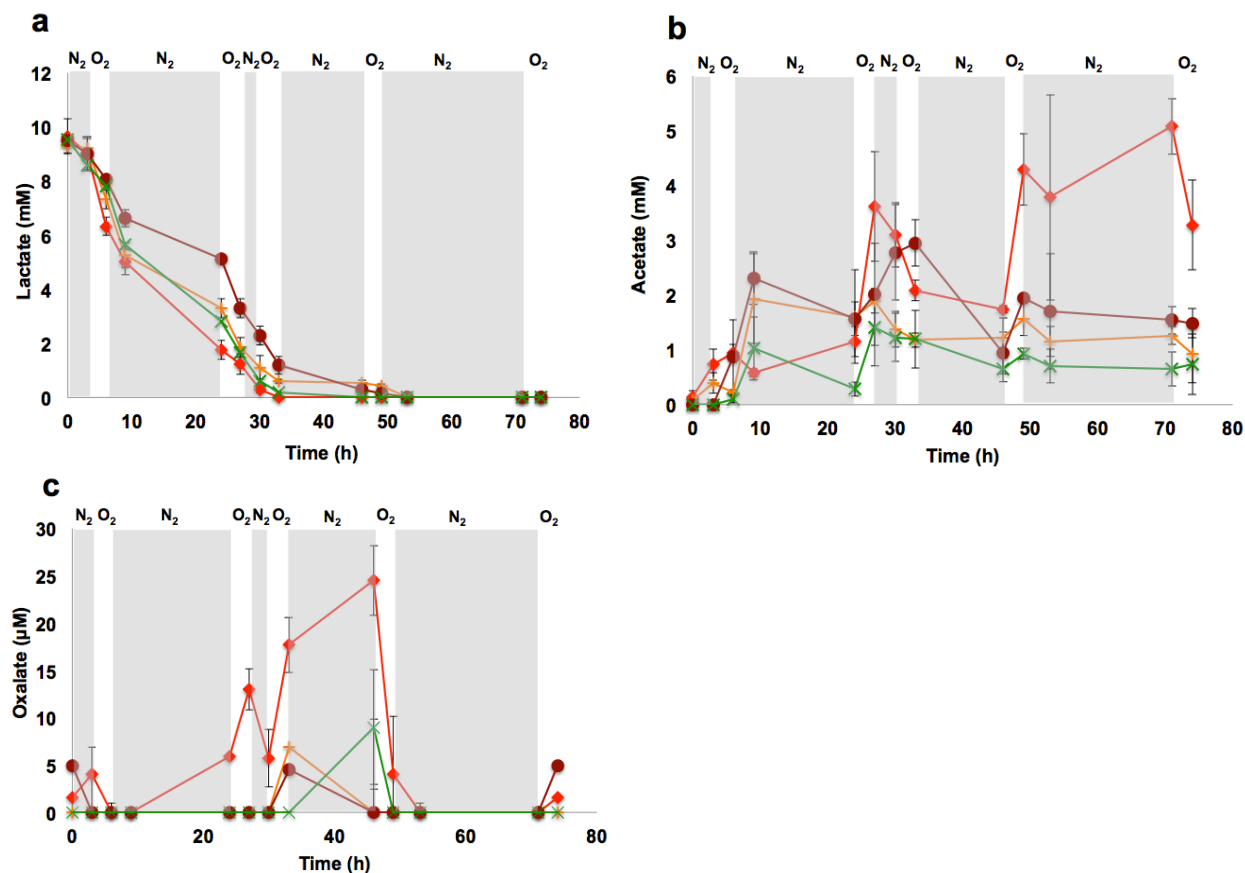


Figure 6. Lactate, acetate, and oxalate concentrations detected during the microbially-driven Fenton degradation of 1,4-dioxane (10 mM initial concentrations) with an aerobic/anaerobic cycling period of 3 h in control incubations: (a) lactate; (b) acetate; (c) oxalate; red $\blacklozenge$, *S. oneidensis* cells + 1,4-dioxane + Fe(III); orange $+$, mannitol; brown $\bullet$, thiourea; green $\times$, nitrate. Grey shaded areas correspond to anaerobic phases and unshaded areas correspond to aerobic phases. Incubations were carried out in two parallel yet identical cultures and error bars indicate range of error between incubations.

A carbon mass balance was also carried out to account for total carbon input and output during the microbially-driven Fenton reaction. Total carbon input under DD conditions (except in controls where 1,4-dioxane was omitted) was 70 mmol. Total carbon output (acetate plus oxalate) under DD conditions was 15.4 mmol and the corresponding carbon input from 1,4-dioxane degradation was 43.2 mmol. The 2-to-4-fold greater amounts of 1,4-dioxane degraded under DD conditions were reflected in a 2-to-3-fold greater total carbon output (acetate plus oxalate)

compared to the DD control incubations carried out in the absence of 1,4-dioxane or in the presence of 1,4-dioxane and the HO[•] scavenging compounds mannitol or thiourea (Table 2). Similar observations were made under DD conditions with aerobic/anaerobic cycling frequencies of 1.5 h, 45 min and 6 h (Supplementary Table S2). Total carbon input (lactate plus 1,4-dioxane) did not completely account for total carbon output (acetate plus oxalate), most likely due to further reaction of acetate and oxalate with HO[•] radicals and the resulting unaccounted carbon loss, potentially as CO₂ (Table 2).

	3 h cycling			
	Input Carbon (mmol)		Output Carbon (mmol)	
	Lactate 1,4-Dioxane	+ 1,4-Dioxane degraded	Acetate	Oxalate
Cells+1,4-dioxane+Fe(III)	70	43.2 ± 2.5	15.4 ± 1.6	0.08 ± 0.004
Cells omitted	70	25.2 ± 1.6	0	0
1,4-Dioxane omitted	30	0	9.6 ± 0.8	0.02 ± 0.004
Fe(III) omitted	70	24 ± 0.7	7.8 ± 0.8	0.06 ± 0.008
Mannitol	70	11 ± 2	5.6 ± 0.6	0.02 ± 0.018
Thiourea	70	12 ± 0.04	9.4 ± 1.2	0.02 ± 0.008
Nitrate	70	11.2 ± 9.6	5 ± 0.6	0.02 ± 0.012

Table 2. Total input and output carbon detected during the microbially-driven Fenton degradation of 1,4-dioxane. Incubations were carried in two parallel yet identical cultures and error bars indicate range of error between incubations. Carbon mmols per liter of reaction volume

The inability of the microbially-driven Fenton reaction to degrade 1,4-dioxane in the presence of HO[•] radical scavenging compounds mannitol or thiourea or in the absence of either *S. oneidensis* cells or Fe(III) implicated HO[•] radicals as the main driver of the 1,4-dioxane degradation process. The absence of 1,4-dioxane degradation under strictly aerobic or strictly anaerobic Fe(III)-reducing conditions indicates that 1,4-dioxane degradation was not due to aerobic or anaerobic enzymatic 1,4-dioxane degradation reactions catalyzed by *S. oneidensis*.^{2, 19} Alternately, the microbially-driven Fenton reaction described in the present study may be initiated by H₂O₂-catalyzed Fe(III) reduction reactions (48, 49). In this scenario, H₂O₂ produced by *S. oneidensis* during the preceding aerobic cycling period catalyzes chemical (purely abiotic) Fe(III) reduction reactions. The H₂O₂-catalyzed Fe(III) reduction reactions, however, are Fe(III) speciation-dependent, and some soluble organic-Fe(III) complexes such as Fe(III)-oxalate are not reduced by H₂O₂ (50). Since *S. oneidensis* produces an yet unidentified Fe(III)-chelating organic ligand that enhances Fe(III) bioavailability (51-53), the results of the present study do not preclude the possibility that soluble organic-Fe(III) complexes produced by *S. oneidensis* are reduced by H₂O₂ produced during the preceding aerobic cycling period. However, the absence of 1,4-dioxane degradation in Fe(III)-amended DD control incubations held under strictly aerobic conditions for 74 h indicates that HO[•] radical production is most likely not initiated by H₂O₂-catalyzed Fe(III) reduction reactions, but requires *S. oneidensis*-catalyzed Fe(III) reduction reactions during the anaerobic cycling periods (48, 49).

Although the LC and IC methods used in the present study may have missed detection of other 1,4-dioxane degradation products (e.g., aldehydes) analyzed by other methods (36), acetate

and oxalate were detected as 1,4-dioxane degradation products of the microbially driven Fenton reaction. In conventional (purely abiotic) Fenton reactions, acetate and oxalate are produced by HO[•] radical attack of 1,4-dioxane with α -oxy radicals as the key intermediate for subsequent degradation reactions (36). The α -oxy radicals follow two separate degradation pathways that depend on O₂ concentrations in the chemical Fenton reaction system. In O₂-limited systems, the α -oxy radicals are degraded to acetate, formate, formaldehyde, and oxalate, while in O₂-replete systems, the α -oxy radicals are degraded to ethylene glycol diformate (24, 36). In the present study, 1,4-dioxane degradation by the microbially driven Fenton reaction resulted in production of acetate and oxalate as the main 1,4-dioxane degradation products, while ethylene glycol diformate was not detected. In addition, formate, glycolate, and glyoxylate were not detected during the microbially-driven 1,4-dioxane degradation process, most likely due to the high reactivity of these intermediates with HO[•] radical³⁶. Since only 40 μ M oxalate was produced during degradation of 10 mM 1,4-dioxane, the majority of 1,4-dioxane may have been mineralized to CO₂ or stalled at another upstream intermediate such as hydroxyethoxyacetic acid (HEAA) (36, 54).

1E. Conclusions and Implications for Future Research/ Implementation. In previous applications of conventional (purely abiotic) Fenton reactions, 1,4-dioxane was degraded at rates approximately 20-fold greater than the microbially-driven Fenton reaction designed in the present study (rate constant of 0.0036 min⁻¹ for 3 h aerobic/anaerobic cycling period at pH 7.0). Such differences may be due to the high concentrations (15 mM) of exogenous H₂O₂ employed to drive the chemical Fenton reaction systems (24, 36). However, after normalization on a per mg protein basis, the reaction rates observed in the present study are 4-to-5-fold greater than the enzymatic rates of 1,4-dioxane degradation by *Pseudonocardia* species (2). In the present study, *S. oneidensis* produced micromolar levels of H₂O₂, presumably as a by-product of microbial aerobic respiration during the aerobic cycling periods. Addition of exogenous H₂O₂ was therefore not required to drive the microbially-driven Fenton reaction. The chemical Fenton reaction also requires re-reduction of Fe(III) produced during H₂O₂-catalyzed Fe(II) oxidation reactions. Fe(III) re-reduction processes such as those catalyzed by UV irradiation are possible during ex situ 1,4-dioxane degradation processes (39), yet UV light penetration represents a formidable obstacle for in situ 1,4-dioxane remediation technologies.

In the microbially-driven Fenton reaction designed in the present study, *S. oneidensis* respiratory processes catalyze both H₂O₂ production and Fe(III) re-reduction. Since microbial Fe(III) reduction has been detected in a variety of aquatic environments, including contaminated subsurface aquifers (55), the microbially-driven Fenton reaction may be induced by exposing Fe(III)-reducing facultative anaerobes in Fe(III)-containing contaminated environments to alternating aerobic and anaerobic conditions. Alternately, the microbially-driven Fenton reaction may be stimulated by injecting soluble Fe(III), nanoparticulate zero-valent iron (nZVI) (56) or electrolytic systems (57) in the flow path of 1,4-dioxane-contaminated subsurface aquifers and exposing endogenous (or injected) Fe(III)-reducing bacteria to alternating aerobic and anaerobic conditions. Microbially-driven Fenton reaction may also be initiated through bioaugmentation at plume fringes where heterogeneous aerobic/anaerobic redox zonation may occur (58). The microbially-driven Fenton reaction thus provides a foundation for development of alternative in situ remediation technologies that degrade 1,4-dioxane or other environmental contaminants susceptible to attack by HO[•] radicals generated by the Fenton reaction.

1F. Literature Cited.

1. Stickney, J. A.; Sager, S. L.; Clarkson, J. R.; Smith, L. A.; Locey, B. J.; Bock, M. J.; Hartung, R.; Olp, S. F., An updated evaluation of the carcinogenic potential of 1,4-dioxane. *Reg. Tox. Pharm* 2003, 38, 183-195.
2. Mahendra, S.; Alvarez-Cohen, L., Kinetics of 1,4-dioxane biodegradation by monooxygenase-expressing bacteria. *Environ Sci Technol* 2006, 40, (17), 5435-42.
3. DeRosa, C. T.; Wilbur, S.; Holler, J.; Richter, P.; Stevens, Y. W., Health evaluation of 1,4-dioxane. *Toxicology and industrial health* 1996, 12, (1), 1-43.
4. Mohr, T. K. G.; Stickney, J. A.; DiGuseppi, W. H., *Environmental Investigation and Remediation: 1,4-Dioxane and other Solvent Stabilizers*. CRC Press: 2010.
5. U.S. Environmental Protection Agency, Integrated Risk Information System (IRIS) on 1,4-Dioxane. In National Center for Environmental Assessment, Washington, DC, 1999.
6. Abe, A., Distribution of 1,4-dioxane in relation to possible sources in the water environment. *Sci Total Environ* 1999, 227, (1), 41-7.
7. Jackson, R. E.; Dwarkanath, V., Chlorinated degreasing solvents: physical-chemical properties affecting aquifer contaminants and remediation. *Ground Water Monit Rem* 1999, 19, 102-110.
8. Lanigan, R. S., Addendum to the final report on the safety assessment of polysorbates. *Int J Toxicol* 2000, 19, 43-89.
9. Zenker, M. J.; Borden, R. C.; Barlaz, M. A., Occurrence and treatment of 1,4-dioxane in aqueous environments. *Environ Eng Sci* 2003, 20, (423-432).
10. Sei, K.; Kakinoki, T.; Inoue, D.; Soda, S.; Fujita, M.; Ike, M., Evaluation of the biodegradation potential of 1,4-dioxane in river, soil and activated sludge samples. *Biodegradation* 2010, 21, (4), 585-91.
11. Adams, C. D.; Scanian, P. A.; Secrist, N. D., Oxidation and biodegradability enhancement of 1,4-dioxane using hydroxide peroxide and ozone. *Environ. Sci. Technol* 1998, 28, 1812-1816.
12. Hill, R. R.; Jeffs, G. E.; Roberts, D. R., Photocatalytic degradation of 1,4-dioxane in aqueous solution. *J Photochem Photobiol A* 1997, 108, 55-58.
13. Beckett, M. A.; Hua, I., Enhanced sonochemical decomposition of 1,4-dioxane by ferrous iron. *Water Research* 2003, 37, 2372-2376.
14. Roy, D.; Anagnostu, G.; Chaphalkar, P., Biodegradation of dioxane and diglyme in industrial waste. *J. Environ. Sci. Health. Part A Environ. Sci. Eng* 1994, 29, 129-147.
15. Chidambara Raj, C. B.; Ramkumar, N.; Haja jahabar siraj, A.; Chidambaram, S. P., Biodegradation of acetic, benzoic, isophthalic, toluic and terephthalic acids using a mixed culture: effluents of PTA production. *Process saf. Environ. Prot* 1997, 75, 245-256.
16. Vainberg, S.; McClay, K.; Masuda, H.; Root, D.; Condee, C.; Zylstra, G. J.; Steffan, R. J., Biodegradation of ether pollutants by *Pseudonocardia* sp. strain ENV478. *Applied and environmental microbiology* 2006, 72, (8), 5218-24.
17. Nakamiya, K.; Hashimoto, S.; Ito, H.; Edmonds, J. S.; Morita, M., Degradation of 1,4-dioxane and cyclic ethers by an isolated fungus. *Applied and environmental microbiology* 2005, 71, (3), 1254-8.
18. Kim, Y. M.; Jeon, J. R.; Murugesan, K.; Kim, E. J.; Chang, Y. S., Biodegradation of 1,4-dioxane and transformation of related cyclic compounds by a newly isolated *Mycobacterium* sp. PH-06. *Biodegradation* 2009, 20, (4), 511-519.

19. Mahendra, S.; Alvarez-Cohen, L., *Pseudonocardia dioxanivorans* sp. nov., a novel actinomycete that grows on 1,4-dioxane. *International journal of systematic and evolutionary microbiology* 2005, 55, (Pt 2), 593-8.
20. Steffan, R., Biodegradation of 1,4-Dioxane. *SERDP* 2007, August.
21. Richard J. Watts; Brett C. Bottenberg; Thomas F. Hess; Mark D. Jensen; Teel., A. L., Role of reductants in the enhanced desorption and transformation of chloroaliphatic compounds by modified Fenton's reactions. *Environ Sci Technol* 1999, 33, 3432-3437.
22. Kinne, M.; Poraj-Kobielska, M.; Ralph, S. A.; Ullrich, R.; Hofrichter, M.; Hammel, K. E., Oxidative cleavage of diverse ethers by an extracellular fungal peroxygenase. *J Biol Chem* 2009, 284, (43), 29343-9.
23. Jho, E. H.; Singhal, N.; Turner, S., Fenton degradation of tetrachloroethene and hexachloroethane in Fe(II) catalyzed systems. *J Hazard Mater* 2010, 184, (1-3), 234-40.
24. Vescovi, T.; Coleman, H. M.; Amal, R., The effect of pH on UV-based advanced oxidation technologies--1,4-dioxane degradation. *J Hazard Mater* 2010, 182, (1-3), 75-9.
25. Kim, Y. K.; Huh, I. R., Enhancing biological treatability of landfill leachate by chemical oxidation. *Environmental Engineering Science* 1997, 14, (1), 73-79.
26. Tyre, B. W.; Watts, R. J.; Miller, G. C., Treatment of 4 Biorefractory Contaminants in Soils Using Catalyzed Hydrogen-Peroxide. *J Environ Qual* 1991, 20, (4), 832-838.
27. Topudurti, K.; Keefe, M.; Wooliever, P.; Lewis, N., Field-Evaluation of Perox-Pure(Tm) Chemical Oxidation Technology. *Water Science and Technology* 1994, 30, (7), 95-104.
28. Andreozzi, R.; Campanella, L.; Frayse, B.; Garric, J.; Gonnella, A.; Giudice, R. L.; Marotta, R.; Pinto, G.; Pollio, A., Effects of advanced oxidation processes (AOPs) on the toxicity of a mixture of pharmaceuticals. *Water Sci Technol* 2004, 50, (5), 23-8.
29. Barbeni, M.; Minero, C.; Pelizzetti, E.; Borgarello, E.; Serpone, N., Chemical Degradation of Chlorophenols with Fenton Reagent (Fe-2++H₂O₂). *Chemosphere* 1987, 16, (10-12), 2225-2237.
30. McKinzi, A. M.; Dichristina, T. J., Microbially driven Fenton reaction for transformation of pentachlorophenol. *Environ Sci Technol* 1999, 33, (11), 1886-1891.
31. Tsai, T. T.; Kao, C. M.; Surampalli, R. Y.; Weng, C. H.; Liang, S. H., Treatment of TCE-Contaminated Groundwater Using Fenton-Like Oxidation Activated with Basic Oxygen Furnace Slag. *J Environ Eng-Asce* 2010, 136, (3), 288-294.
32. Pignatello, J. J.; Liu, D.; Huston, P., Evidence for an Additional Oxidant in the Photoassisted Fenton Reaction. *Environ Sci Technol* 1999, 33, 1832-1839.
33. Yeh, C. K. J.; Wu, H. M.; Chen, T. C., Chemical oxidation of chlorinated non-aqueous phase liquid by hydrogen peroxide in natural sand systems. *J Hazard Mater* 2003, 96, (1), 29-51.
34. Baciocchi, R.; Boni, M. R.; D'Aprile, L., Application of H₂O₂ lifetime as an indicator of TCE Fenton-like oxidation in soils. *J Hazard Mater* 2004, 107, (3), 97-102.
35. Son, H. S.; Im, J. K.; Zoh, K. D., A Fenton-like degradation mechanism for 1,4-dioxane using zero-valent iron (Fe⁰) and UV light. *Water Research* 2009, 43, (5), 1457-1463.
36. Stefan, M. I.; Bolton, J. R., Mechanism of the degradation of 1,4-dioxane in dilute aqueous solution using the UV hydrogen peroxide process. *Environ Sci Technol* 1998, 32, (11), 1588-1595.
37. Kim, S. M.; Vogelpohl, A., Degradation of organic pollutants by the photo-fenton-process. *Chem Eng Technol* 1998, 21, (2), 187-191.
38. Coleman, H. M.; Vimonses, V.; Leslie, G.; Amal, R., Degradation of 1,4-dioxane in water using TiO₂ based photocatalytic and H₂O₂/UV processes. *J Hazard Mater* 2007, 146, (3), 496-501.

39. Kim, C. G.; Seo, H. J.; Lee, B. R., Decomposition of 1,4-dioxane by advanced oxidation and biochemical process. *Journal of environmental science and health. Part A, Toxic/hazardous substances & environmental engineering* 2006, 41, (4), 599-611.
40. Dichristina, T. J.; Delong, E. F., Isolation of Anaerobic Respiratory Mutants of *Shewanella-Putrefaciens* and Genetic-Analysis of Mutants Deficient in Anaerobic Growth on Fe³⁺. *J Bacteriol* 1994, 176, (5), 1468-1474.
41. Dichristina, T. J.; Arnold, R. G.; Lidstrom, M. E.; Hoffmann, M. R., Dissimilative Iron Reduction by the Marine Eubacterium *Alteromonas putrefaciens* Strain 200. *Water Science & Technology* 1988, 20, (11-12), 69-79.
42. DiChristina, T. J.; Moore, C. M.; Haller, C. A., Dissimilatory Fe(III) and Mn(IV) reduction by *Shewanella putrefaciens* requires ferE, a homolog of the pulE (gspE) type II protein secretion gene. *J Bacteriol* 2002, 184, (1), 142-51.
43. Sambrook, J.; Fritsh, E. F.; Maniatis, T., *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 1989.
44. Obuekwe, C. O.; Westlake, D. W. S.; Cook, F. D., Effect of Nitrate on Reduction of Ferric Iron by a Bacterium Isolated from Crude-Oil. *Can J Microbiol* 1981, 27, (7), 692-697.
45. Dichristina, T. J., Effects of Nitrate and Nitrite on Dissimilatory Iron Reduction by *Shewanella-Putrefaciens*-200. *J Bacteriol* 1992, 174, (6), 1891-1896.
46. Kiassen, N. V.; Marchington, D.; McGowan, H. C. E., H₂O₂ Determination by the I₃-Method and by KMnO₄ Titration. *Anal. Chem* 1994, 66, 2921-2925.
47. Nakamura, S.; Daishima, S., Simultaneous determination of 22 volatile organic compounds, methyl-tert-butyl ether, 1,4-dioxane, 2-methylisoborneol and geosmin in water by headspace solid phase microextraction-gas chromatography-mass spectrometry. *Anal Chim Acta* 2005, 548, (1-2), 79-85.
48. Laat, J. D.; Gallard, H., Catalytic Decomposition of Hydrogen Peroxide by Fe(III) in Homogeneous Aqueous Solution: Mechanism and Kinetic Modeling. *Environ. Sci. Technol* 1999, 33, 2726-2732.
49. Halliwell, B.; Gutteridge, J. M., Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Archives of biochemistry and biophysics* 1986, 246, (2), 501-14.
50. Daneshvar, N.; Khataee, A. R., Removal of azo dye C.I. acid red 14 from contaminated water using Fenton, UV/H(2)O(2), UV/H(2)O(2)/Fe(II), UV/H(2)O(2)/Fe(III) and UV/H(2)O(2)/Fe(III)/oxalate processes: a comparative study. *Journal of environmental science and health. Part A, Toxic/hazardous substances & environmental engineering* 2006, 41, (3), 315-28.
51. Jones, M. E.; Fennessey, C. M.; DiChristina, T. J.; Taillefert, M., *Shewanella oneidensis* MR-1 mutants selected for their inability to produce soluble organic-Fe(III) complexes are unable to respire Fe(III) as anaerobic electron acceptor. *Environmental microbiology* 2010, 12, (4), 938-50.
52. Fennessey, C. M.; Jones, M. E.; Taillefert, M.; DiChristina, T. J., Siderophores Are Not Involved in Fe(III) Solubilization during Anaerobic Fe(III) Respiration by *Shewanella oneidensis* MR-1. *Applied and environmental microbiology* 2010, 76, (8), 2425-2432.
53. Taillefert, M.; Beckler, J. S.; Carey, E.; Burns, J. L.; Fennessey, C. M.; DiChristina, T. J., *Shewanella putrefaciens* produces an Fe(III)-solubilizing organic ligand during anaerobic respiration on insoluble Fe(III) oxides. *J Inorg Biochem* 2007, 101, (11-12), 1760-1767.

54. Mahendra, S.; Petzold, C. J.; Baidoo, E. E.; Keasling, J. D.; Alvarez-Cohen, L., Identification of the intermediates of in vivo oxidation of 1,4-dioxane by monooxygenase-containing bacteria. *Environ Sci Technol* 2007, 41, (21), 7330-6.
55. Lovley, D. R., Microbial Fe(III) reduction in subsurface environments. *FEMS Microbiology Reviews* 1997, 20, 305-313.
56. Joo, S. H.; Feitz, A. J.; Sedlak, D. L.; Waite, T. D., Quantification of the oxidizing capacity of nanoparticulate zero-valent iron. *Environ Sci Technol* 2005, 39, (5), 1263-8.
57. Gilbert, D. M.; Sale, T. C., Sequential Electrolytic Oxidation and Reduction of Aqueous Phase Energetic Compounds. *Environ Sci. Technol* 2005, 39, 9270-9277.
58. Haack, S. K.; Bekins, B. A., Microbial populations in contaminant plumes. *Hydrogeology* 2000, 8, 63-76.
59. Sekar, R.S.; DiChristina, T.J. Microbially Driven Fenton Reaction for Degradation of the Widespread Environmental Contaminant 1,4-Dioxane. *Environmental Science and Technology* 2014, 48, 12858-12867.

2. Simultaneous Degradation of Comingled Contaminants Trichloroethylene, Tetrachloroethylene, and 1,4-Dioxane by a Microbially Driven Fenton Reaction.

2A. Objective. The main objectives of the present study were to (i) design a new fed batch, microbially-driven Fenton reaction system that minimizes contaminant loss due to volatility by separating the Fe(II)-generating, H₂O₂-generating, and contaminant degradation phases and ii) apply the new microbially-driven Fenton reaction system to simultaneously degrade single, binary, and ternary mixtures of TCE, PCE, and 1,4-dioxane.

2B. Background. The chlorinated organic solvents trichloroethylene (TCE) and tetrachloroethylene (PCE) have been historically employed as solvents in a variety of industrial processes including vapor degreasing of metal surfaces, paint stripping, and dry cleaning (1, 2). TCE and PCE are carcinogenic and improper disposal practices at industrial sites have resulted in widespread contamination of soil and ground water (1-6). Due to their high density and low aqueous phase solubility, TCE and PCE are also highly persistent in contaminated environments (3, 4). The potential carcinogen 1,4-dioxane is generally employed as a stabilizer for TCE and PCE in industrial processes (7-11), and thus TCE- and PCE-contaminated ground water is often comingled with 1,4-dioxane (9, 12-20).

Current TCE and PCE remediation technologies are based on photolysis, sonolysis, and reductive transformations by iron-bearing minerals or dechlorinating microorganisms (6, 21-26). Photolysis and sonolysis, however, are limited by UV light penetration and low energy transfer efficiency, respectively (27), while microbially-catalyzed reductive dehalogenation reactions are limited by microbial nutrient requirements, contaminant bioavailability, and incomplete dechlorination leading to the production of toxic intermediates (28). Chemical oxidation is also an attractive remediation technology for degradation of TCE, PCE, and 1,4-dioxane (2, 5, 12, 33). Currently deployed chemical oxidants include ozone, titanium oxide, persulfate, permanganate, peroxide, zero valent iron, and hydroxyl (HO[•]) radicals (2, 5). HO[•] radicals represent a powerful oxidant with oxidation potentials (+2.76 V) higher than ozone (+2.07 V), persulfate (+2.01 V), and permanganate (+1.68 V). (33) HO[•] radicals may be produced by the conventional Fenton reaction (eq 1). Conventional Fenton reaction-driven degradation of TCE, PCE, and 1,4-dioxane is limited, however, by the high concentrations of the Fenton reagents Fe(II) and H₂O₂ that must be continuously supplied to produce HO[•] radicals and drive TCE, PCE, and 1,4-dioxane degradation (1, 24).

Microbially-driven Fenton reactions that alternately produce the Fenton reagents H₂O₂ (via microbial O₂ respiration) and Fe(II) (via microbial Fe(III) reduction) alleviate the need for continual addition of H₂O₂ and Fe(II) to drive HO[•] radical production (12, 17, 29, 41). The Fe(III)-reducing facultative anaerobe *Shewanella oneidensis* was recently employed to drive the Fenton reaction for oxidative degradation of PCP and 1,4-dioxane (29, 36). In the *S. oneidensis*-driven Fenton reaction, batch liquid cultures were amended with Fe(III) and 1,4-dioxane and subsequently exposed to alternating anaerobic and aerobic conditions. During the anaerobic period *S. oneidensis* produced Fe(II) via microbial Fe(III) reduction, while during the aerobic period, *S. oneidensis* produced H₂O₂ via microbial O₂ respiration. During the transition from anaerobic-to-aerobic conditions, Fe(II) and H₂O₂ interacted chemically via the Fenton reaction to produce HO[•] radicals that completely degraded 1,4-dioxane at source zone concentrations (29). Although TCE and PCE are often comingled with 1,4-dioxane in contaminated ground water, only a limited

number of studies have examined simultaneous degradation of multiple contaminants, with emphasis on binary mixtures of TCE and 1,4-dioxane or TCE and PCE (26, 42-44).

2C. Materials and Methods. *Design of Fed Batch Reactor System for Simultaneous Degradation of TCE, PCE, and 1,4-Dioxane by the Microbially-Driven Fenton Reaction.* The toxicity of single, binary, and ternary mixtures of TCE, PCE, and 1,4-dioxane was tested in LS medium by comparing aerobic growth of *S. oneidensis* batch cultures in the presence and absence of the contaminant mixtures. To avoid inadvertent loss of contaminants due to volatility during injection of compressed nitrogen or compressed air (Henry's law constant for TCE: $0.11 \text{ mol kg}^{-1} \text{ bar}^{-1}$, PCE: $0.05 \text{ mol kg}^{-1} \text{ bar}^{-1}$, 1,4-dioxane: $220 \text{ mol kg}^{-1} \text{ bar}^{-1}$) (29, 48-51), the batch reactor system previously employed for 1,4-dioxane degradation (29) was modified and the Fe(II)-generating, H_2O_2 -generating, and contaminant degradation phases were separated (Fig. S14 in (59)). In the Fe(II)-generating phase, contaminant-free *S. oneidensis* liquid cultures ($10^9 \text{ cells mL}^{-1}$ in LS medium amended with 10 mM Fe(III)) were incubated in 60-mL glass serum bottles under anaerobic conditions by injecting (hydrated) compressed nitrogen until the entire 10 mM pool of Fe(III) was reduced to approximately 10 mM Fe(II) (approximately 24 h of anaerobic incubation; Fe(II)-generating phase). The compressed nitrogen line was then replaced by a (hydrated) compressed air line and the 10 mM Fe(II)-containing *S. oneidensis* liquid culture was incubated under aerobic conditions until the 10 mM pool of Fe(II) was oxidized to approximately 4 mM level (approximately 24 h of aerobic incubation; H_2O_2 -generating phase). The 4 mM residual Fe(II) was carried into the contaminant degradation phase to interact with the pool of microbially-produced H_2O_2 and generate $\text{HO}^\bullet$ radicals (similar to $\text{HO}^\bullet$ radical production by the previously reported 1,4-dioxane-degrading batch reactor system) (29).

The contaminant degradation phase was initiated by adding single, binary, and ternary mixtures of TCE, PCE, and 1,4-dioxane with a sterile syringe. The contaminant degradation phase was carried out for approximately 5 days (without gas injection) and the contaminant concentrations were monitored via high pressure liquid chromatography (HPLC). Following completion of the first 5-day contaminant degradation phase (i.e., after contaminants were degraded to below detection limits), a second cycle of Fe(II)-generating and H_2O_2 -generating phases was carried out during the 7-8 and 8-9 day time periods, respectively, followed by re-spiking of contaminant mixtures to initial concentrations and carrying out a second 5-day contaminant degradation phase during the 9-14 day time period. An identical third cycle of Fe(II)-generating, H_2O_2 -generating, and contaminant degradation phases was carried during the 14-19 day time period. Reactor temperature (25°C) and pH (7.0) were held constant throughout all reactor phases.

Inhibition of the Microbially-Driven Fenton Reaction in Control Incubations. A series of four control incubations were carried out to confirm that the single, binary, and ternary mixtures of TCE, PCE, and 1,4-dioxane were degraded by $\text{HO}^\bullet$ radicals generated by the *S. oneidensis*-driven Fenton reaction. The four control incubations were carried out in the fed batch reactor system described above with the following changes: In the first set of control incubations, the H_2O_2 -generating and contaminant degradation phases were carried out with 15 mM NO_3^- replacing 10 mM Fe(III) as electron acceptor. In the second set of control incubations, the $\text{HO}^\bullet$ radical-scavenging compounds mannitol (120 mM) and thiourea (20 mM) were added to the fed batch reactor system prior to initiating the H_2O_2 -generating phase. The toxicities of mannitol and thiourea were tested in LS medium by comparing aerobic growth of *S. oneidensis* batch cultures

in the presence and absence of mannitol or thiourea and single, binary, and ternary mixtures of TCE, PCE, and 1,4-dioxane. In the third set of control incubations, Fe(III) or *S. oneidensis* cells (abiotic control) were omitted from the H₂O₂-generating and contaminant degradation phases. In the fourth set of control incubations, contaminant concentrations were monitored in abiotic sealed serum bottles maintained under strict anaerobic conditions without injection of compressed nitrogen for a 20-day anaerobic incubation period. Analytical methods can be found in detail in the primary literature (61).

2D. Results and Discussion. The Fe(III)-reducing facultative anaerobe *S. oneidensis* was recently employed to drive the Fenton reaction for HO[•] radical production and 1,4-dioxane degradation in a batch reactor system that included *S. oneidensis* cells, Fe(III), and alternate injections of compressed nitrogen (to facilitate microbial Fe(III) reduction) and compressed air (to facilitate microbial H₂O₂ production) (29). The Henry's Law constant for TCE (0.11 mol kg⁻¹ bar⁻¹) and PCE (0.05 mol kg⁻¹), however, are over three orders of magnitude lower than the Henry's law constant for 1,4-dioxane (220 mol kg⁻¹ bar⁻¹) (29, 48-51). Thus, in initial batch reactor designs, the compressed gas injections quickly volatilized TCE and PCE to below detection levels (data not shown), while the concentrations of TCE and PCE remained constant in the absence of compressed gas injection (Figs. S2 and S3). The batch reactor system was therefore modified to avoid TCE and PCE loss due to volatility. In the modified fed batch reactor system, the Fe(II)-generating (microbial Fe(III) reduction stimulated by injection of compressed nitrogen), H₂O₂-generating (microbial H₂O₂ production stimulated by injection of compressed air), and contaminant degradation phases (without injection of compressed gases) were separated and the fed batch reactor system was cycled three times through each of the three separate phases (Fig. S14 in (59)).

In the following results section, the single, binary, and ternary mixtures of TCE, PCE, and 1,4-dioxane were designated as follows: TCE alone = T, PCE alone = P, 1,4-dioxane alone = D, TCE + PCE = TP, TCE + 1,4-dioxane = TD, PCE + 1,4-dioxane = PD, and TCE + PCE + 1,4-dioxane = TPD. To test for TPD toxicity, *S. oneidensis* was grown aerobically in LS growth medium supplemented with a ternary mixture of T (100 μM), P (100 μM), and D (2 mM). Aerobic growth rates in the presence and absence of TPD were nearly identical, which indicated that the TPD concentrations did not inhibit *S. oneidensis* metabolic activity (Fig S1).

The following strategy was applied to all bioreactors: to initiate HO[•] radical production by the *S. oneidensis*-driven Fenton reaction, Fe(III)-containing *S. oneidensis* cultures were incubated under anaerobic conditions for 24 h, the pool of 10 mM Fe(III) was microbially reduced. At the 24-h time point, compressed air was injected for the next 24 h into all incubations, and Fe(II) was air-oxidized. At the 2-d time point, the T, TP, TD, and TPD mixtures were added to initiate the first contaminant degradation phase (2-7 d time period). At the 7-d time point, a second cycle of Fe(II)- and H₂O₂-generating phases was carried out. At the 9-d time point, the T, TP, TD, and TPD mixtures were again added to initiate a second contaminant degradation phase (9-14 d time period). At the 14-d time point, a third cycle of Fe(II)- and H₂O₂-generating phases was carried out. At the 16-d time point, the T, TP, TD, and TPD mixtures were added to initiate a final contaminant degradation phase (16-19 d time period).

TCE Degradation in the Presence and Absence of PCE and 1,4-Dioxane. The rates of TCE degradation (in single, binary, and ternary mixtures of contaminants) ranged from 84-137% between the first, second, and third TCE degradation phases (i.e., by comparing rates of TCE degradation in phases T1, T2, T3 or TP1, TP2, TP3 or TD1, TD2, TD3 or TPD1, TPD2, TPD3; Fig. 2a).

During the first contaminant degradation phase with T as sole contaminant, T was degraded at an initial rate of $1.1 \mu\text{M h}^{-1}$ (Figs. 1b, 4a, and Table S3). By comparison, T was degraded in TP, TD, and TPD mixtures at initial rates 71%, 82%, and 74%, respectively, of the T degradation rate with T as sole contaminant (i.e., % of T1 degradation rate; Figs. 1b, 4a, and Table S3). T was degraded to below detection limits by the end of the first degradation phase (7-d time point) with T as sole contaminant and in the TD, TP, and TPD mixtures (Fig. 1b).

During the second contaminant degradation phase with T as sole contaminant, T was degraded at an initial rate of $1.5 \mu\text{M h}^{-1}$ (Figs. 1b, 4a, and Table S3). By comparison, T was degraded in the TP, TD, and TPD mixtures at initial rates of 51%, 67%, and 55%, respectively, of the T degradation rate with T as sole contaminant (i.e., % of T2 degradation rate; Figs. 1b, 4a, and Table S3). T was degraded to below detection limits by the end of the second degradation phase (14-d time point) with T as sole contaminant and in the TD, TP, and TPD mixtures (Fig. 1b).

During the third contaminant degradation phase with T as sole contaminant, T was degraded in the TP, TD, and TPD mixtures at initial rates of $1.4 \mu\text{M h}^{-1}$ (Figs. 1b, 4a, and Table S3). By comparison, T was degraded at initial rates of 46%, 65%, and 74%, respectively, of the T degradation rate with T as sole contaminant (i.e., % of T3 degradation rate; Figs. 1b, 4a, and Table S3). T was degraded to below detection limits by the end of the third degradation phase (19-d time point) with T as sole contaminant. Due to early termination of the third contaminant degradation phase, T was degraded to 37, 56, and 28 μM levels in the TD, TP, and TPD mixtures, respectively; Fig. 1b).

T was degraded at nearly identical rates as sole contaminant in the three successive contaminant degradation phases (2-7, 9-14, and 16-19 d time periods; Fig. 2a and Table S3). T concentrations remained constant at 100 μM in parallel control incubations lacking Fe(III) or *S. oneidensis* cells (abiotic control; Fig. S2 in (59)), but including three identical cycles of successive anaerobic (Fe(II)-generating), aerobic (H_2O_2 -generating), and contaminant (T, TP, TD, and TPD) degradation phases. Identical patterns of microbial (*S. oneidensis*-catalyzed) Fe(III) reduction and chemical (O_2 -catalyzed) Fe(II) oxidation were observed in parallel control incubations with contaminants omitted (Fig. 1a), which indicated that rates of microbially-catalyzed Fe(III) reduction and O_2 -catalyzed Fe(II) oxidation were not affected by the presence of the T, TP, TD, or TPD mixtures.

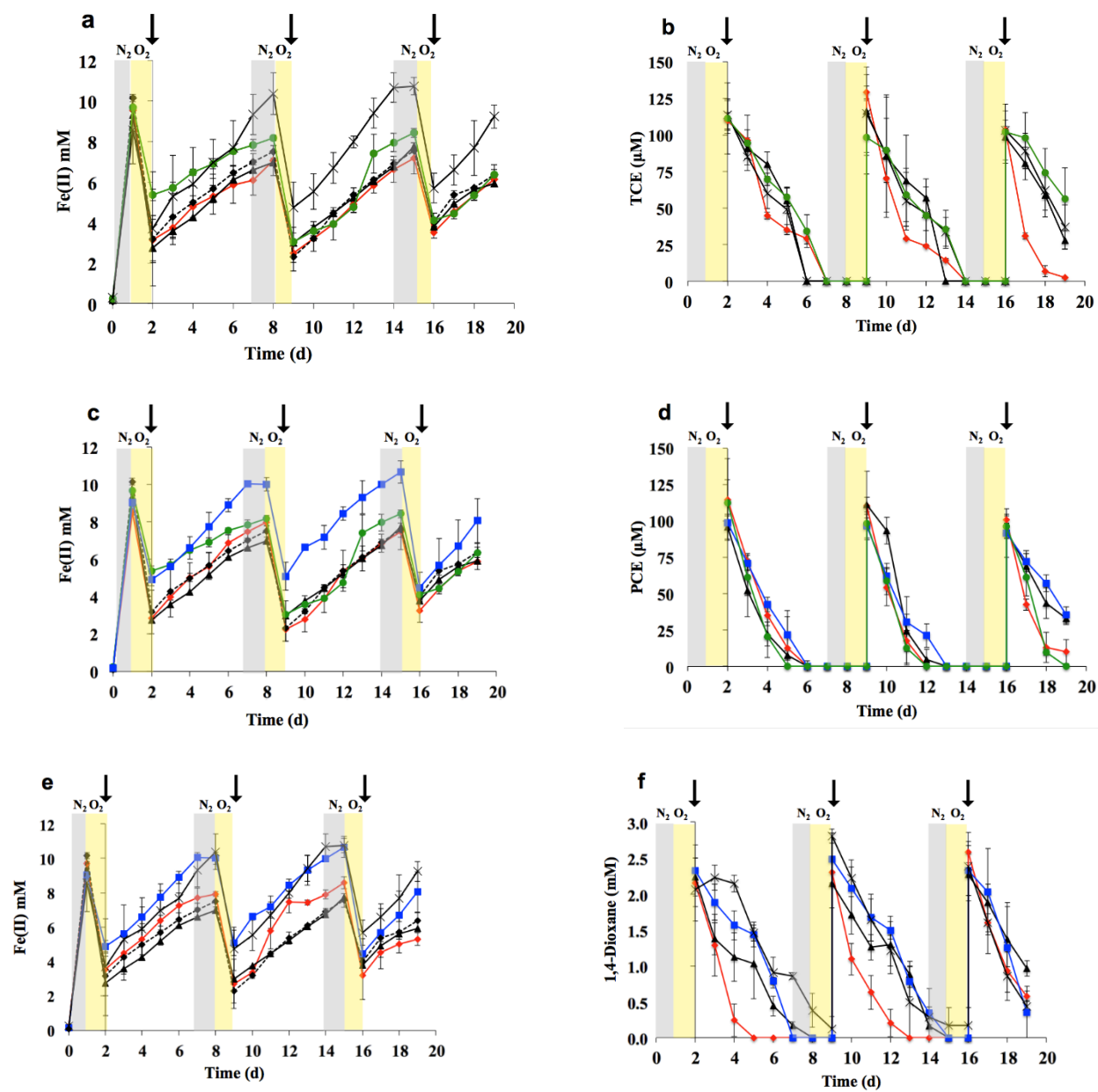


Figure 1. Concentration profiles during microbial Fenton degradation of TCE, PCE and 1,4-dioxane in fed batch liquid cultures of *S. oneidensis* amended with 10 mM Fe(III)-citrate, single, binary, and ternary mixtures of TPD (100 μ M TCE, 100 μ M PCE and 2 mM 1,4-dioxane) and subjected to : Anaerobic (0-1, 7-8, 14-15 day time periods), aerobic (1-2, 8-9, 15-16 day time periods) and TPD degradation (2-7, 9-14, 16-19 day time periods) phases: (a) Fe(II) (for TCE reactions); (b) TCE; (c) Fe(II) (for PCE reactions); (d) PCE; (e) Fe(II) (for 1,4-dioxane reactions); (f) 1,4-dioxane; solid red (◆), single contaminant only; solid black (x), TCE + 1,4-dioxane; solid green (●), TCE + PCE; solid blue (■), PCE + 1,4-dioxane; solid black (▲), TCE + PCE + 1,4-dioxane; dashed black (◆), no contaminant control. Grey shaded areas correspond to Fe(II)-generating phase, yellow shaded areas correspond to H₂O₂-generating phase, and unshaded areas correspond to contaminant degradation phase. Arrows indicate time of addition (day 2) and respiking (day 9 & 16) of contaminants. Error bars represent range of errors in duplicate batch reactors.

PCE Degradation in the Presence and Absence of TCE and 1,4-Dioxane. Nearly identical patterns of microbial (*S. oneidensis*-catalyzed) Fe(III) reduction and chemical (O₂-catalyzed) Fe(II) oxidation were observed in analogous P, TP, PD, and TPD contaminant degradation experiments (Fig. 1c). P was degraded at an initial rate of 1.4 μ M h⁻¹ during the contaminant degradation phase with P as sole contaminant (Figs. 1d, 4b). By comparison, P was degraded in the PT, PD, and TPD mixtures at initial rates of 110%, 75%, and 62%, respectively, of the P degradation rate with P as sole contaminant (i.e., % of P1 degradation rate; Figs. 1d, 4b). P was degraded to below detection limits by the end of the first degradation phase (7-d time point) with P as sole contaminant and in the PT, PD, and TPD mixtures (Fig. 1d).

During the second contaminant degradation phase with P as sole contaminant, P was degraded at an initial rate of 1.6 μ M h⁻¹ (Figs. 1d, 4b, and Table S4). By comparison, P was degraded at initial rates of 88%, 69%, and 96%, respectively, of the P degradation rate with P as sole contaminant (i.e., % of P2 degradation rate; Figs. 1d, 4b, and Table S4). P was degraded to below detection limits by the end of the second degradation phase (14-d time point) with P as sole contaminant and in the PT, PD, and TPD mixtures (Fig. 1d).

During the third contaminant degradation phase with P as sole contaminant, P was degraded at an initial rate of 1.2 μ M h⁻¹ (Figs. 1d, 4b). By comparison, P was degraded at initial rates of 98%, 69%, and 72%, respectively, of the P degradation rate with P as sole contaminant (i.e., % of P3 degradation rate; Figs. 1d, 4b). P was degraded to below detection limits by the end of the third degradation phase (19-d time point) with PT as contaminant mixture. Due to early termination of the third contaminant degradation phase (19-d time point), P was degraded to 10, 36, and 33 μ M levels in the P, PD, and TPD mixtures, respectively; Fig. 1d).

P was degraded as sole contaminant at nearly identical rates in the three successive contaminant degradation phases (2-7, 9-14, and 16-19 day time periods; Fig. 2b and Table S4). P concentrations remained constant at 100 μ M in parallel control incubations lacking Fe(III) or *S. oneidensis* cells (abiotic control; Fig. S3 in (59)), but including three identical cycles of successive anaerobic (Fe(II)-generating), aerobic (H₂O₂-generating), and contaminant (P, PT, PD, and TPD) degradation phases. Identical patterns of microbial (*S. oneidensis*-catalyzed) Fe(III) reduction and chemical (O₂-catalyzed) Fe(II) oxidation were observed in parallel control incubations with contaminants omitted (Fig. 1c), which indicated that rates of microbially-catalyzed Fe(III) reduction and O₂-catalyzed Fe(II) oxidation were not affected by the presence of the P, PT, PD, or TPD mixtures.

1,4-Dioxane Degradation in the Presence and Absence of TCE And PCE. Nearly identical patterns of microbial (*S. oneidensis*-catalyzed) Fe(III) reduction and chemical (O₂-catalyzed Fe(II)

oxidation were observed in analogous D, DT, DP, and TPD contaminant degradation experiments (Figs. 1e). At the 2-d time point, the D, DT, DP and TPD mixtures were added to initiate the first contaminant degradation phase (2-7 d time period). D was degraded at an initial rate of $30.8 \mu\text{M h}^{-1}$ (Figs. 1f, 4c, and Table S5). By comparison, D was degraded in the DT, DP, and TPD mixtures at initial rates of 35%, 39%, and 54%, respectively, of the D degradation rate with D as sole contaminant (i.e., % of D1 degradation rate; Figs. 1f, 4c, and Table S5). D was degraded to below detection limits by the end of the first degradation phase (7-d time point) in the D and DP mixtures. However, D was only degraded to 0.17 mM and 0.85 mM levels in the DT and TPD mixtures, respectively (Fig. 1f).

During the second contaminant degradation phase with D as sole contaminant, D was degraded at an initial rate of $36.2 \mu\text{M h}^{-1}$ (Figs. 1f, 4c, and Table S5). By comparison, D was degraded at initial rates of 61%, 38%, and 36%, respectively, of the D degradation rate with D as sole contaminant (i.e., % of D2 degradation rate; Figs. 1f, 4c, and Table S5). D was degraded to below detection limits by the end of the second degradation phase (14-d time point) with D as sole contaminant. However, D was degraded only to 0.34, 0.30, and 0.16 mM levels in the DT, DP, and TPD mixtures, respectively (Fig. 1f).

During the third contaminant degradation phase with D as sole contaminant, D was degraded at an initial rate of $27.9 \mu\text{M h}^{-1}$ (Figs. 1f, 4c, and Table S5). By comparison, D was degraded at initial rates of 97%, 101%, and 70%, respectively, of the D degradation rate with D as sole contaminant (i.e., % of D3 degradation rate; Figs. 1f, 4c, and Table S5). Due to early termination of the third degradation phase (19-d time point), D was degraded to 0.6, 0.43, 0.48, and 0.96 mM levels in the D, DT, DP, and TPD mixtures, respectively (Fig. 1f).

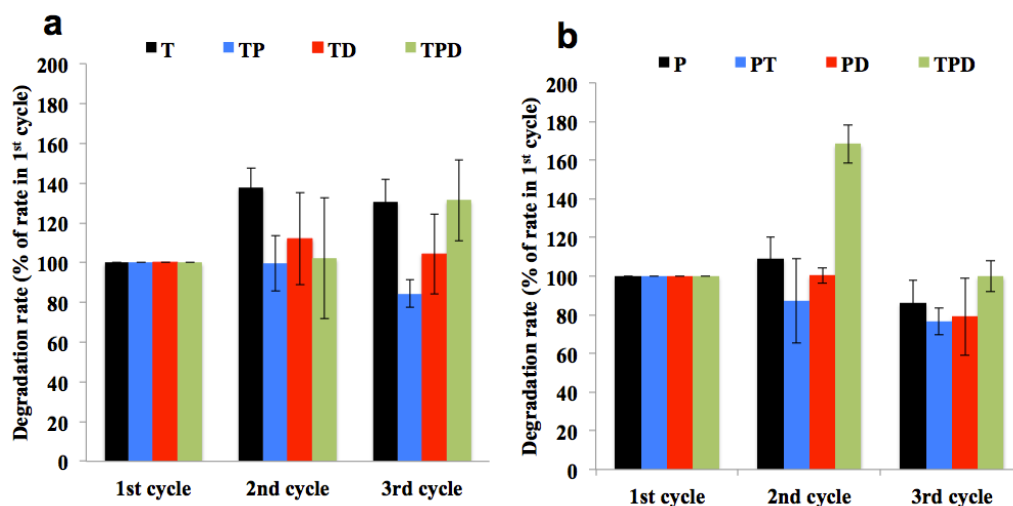
D was degraded at nearly identical rates as sole contaminant in the three successive contaminant degradation phases (2-7, 9-14, and 16-19 d time periods; Fig. 2c and Table S5). D concentrations remained constant at $100 \mu\text{M}$ in parallel control incubations lacking Fe(III) or *S. oneidensis* cells (abiotic control; Fig. S4 in (59)), but including three identical cycles of successive anaerobic (Fe(II)-generating), aerobic (H_2O_2 -generating), and contaminant (D, DT, DP, and TPD) degradation phases. Identical patterns of microbial (*S. oneidensis*-catalyzed) Fe(III) reduction and chemical (O_2 -catalyzed) Fe(II) oxidation were observed in parallel control incubations with contaminants omitted (Fig. 1e), which indicated that rates of microbially-catalyzed Fe(III) reduction and O_2 -catalyzed Fe(II) oxidation were not affected by the presence of the D, DT, DP, or TPD mixtures.

Inhibition of the Microbially-Driven Fenton Reaction in Control Incubations. To confirm that the single, binary, and ternary mixtures of TPD were degraded by $\text{HO}^\cdot$ radicals generated by the *S. oneidensis*-driven Fenton reaction, a series of control incubations were carried out in the presence of the $\text{HO}^\cdot$ radical-scavenging compounds mannitol or thiourea. The single, binary, or ternary mixtures of TPD were not degraded in the contaminant degradation phase in the presence of mannitol or thiourea (Figs. S2-4). To test for toxicity of mannitol and thiourea in the presence of TPD, *S. oneidensis* cultures were grown aerobically in LS growth medium supplemented with mannitol or thiourea and the ternary TPD mixture. Aerobic growth rates in the presence or absence of mannitol or thiourea were nearly identical, which indicates that a combination of mannitol or thiourea and TPD did not inhibit *S. oneidensis* metabolic activity (Fig. S1 in (59)). The ability of the $\text{HO}^\cdot$ radical-scavenging compounds mannitol and thiourea to inhibit TPD degradation indicates that $\text{HO}^\cdot$ radicals derived from the microbially-driven Fenton reaction are involved in the degradation of single, binary, and ternary TPD mixtures.

The requirement for microbial Fe(III) reduction was tested by replacing Fe(III) with NO_3^- and carrying out an otherwise identical set of TPD degradation experiments with NO_3^- -containing fed batch reactors. TPD was not degraded with NO_3^- as electron acceptor (Figs. S2-4), or in the absence of *S. oneidensis* cells (Figs. S2-4). These results indicated that microbial Fe(III) reduction was required to drive the TPD degradation process. Furthermore, in all TPD degradation and control incubations, lactate was consumed at similar rates ($87.5 - 100.0 \mu\text{M h}^{-1}$), while lactate concentrations remained constant during the 17-day abiotic control incubations (Figs. S6-8 and S12). Due to the microbially-catalyzed aerobic oxidation of lactate during the aerobic (H_2O_2 -generating) phase, acetate was produced at rates of $50.0 - 66.7 \mu\text{M h}^{-1}$ (Figs. S9-11 and S13).

Although TCE and PCE are often comingled with 1,4-dioxane in contaminated soil and ground water, only a limited number of studies have examined simultaneous degradation of multiple contaminants, with emphasis on binary mixtures of TCE and 1,4-dioxane or TCE and PCE (26, 42-44). The present study is the first report of simultaneous degradation of source zone levels of ternary mixtures of TCE, PCE, and 1,4-dioxane. In control incubations held under strictly anaerobic Fe(III)-reducing conditions, the TPD mixtures were not degraded, which demonstrated that *S. oneidensis* was unable to enzymatically degrade TCE, PCE, or 1,4-dioxane in the contaminant degradation phase. In addition, under a normal cycle of alternating anaerobic and aerobic conditions, the three contaminant mixtures were not degraded in the absence of Fe(III), in the absence of *S. oneidensis* cells, or in the presence of the $\text{HO}^\cdot$ radical-scavenging compounds mannitol or thiourea. These findings indicate that the TPD mixtures were simultaneously degraded by $\text{HO}^\cdot$ radicals produced by the microbially-driven Fenton reaction (14).

In a similar fashion, the rates of PCE and 1,4-dioxane degradation (in single, binary, and ternary mixtures of contaminants) also ranged from 77-168%. However, during the second (DT mixture) and third (DT and DP mixtures) 1,4-dioxane degradation cycles, 1,4-dioxane degradation rates were 104%, 150%, and 133% higher, respectively. The higher 1,4-dioxane degradation rates were most likely due to the incomplete degradation of 1,4-dioxane during the preceding contaminant degradation phase, the resulting carry over of 1,4-dioxane to the next cycle, and the inadvertent higher ratios of D/T or D/P (second phase: D/T - 33% higher; third phase: D/T - 28% higher, D/P - 6% higher) compared to the respective D/T or D/P ratios of the first degradation phase (Figs. 1b, 1d, 1f, 2b, 2c, and Tables S4 and S5).



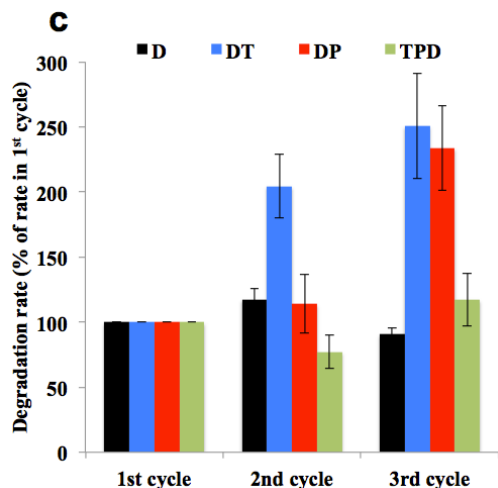


Figure 2. Comparison of rate of degradation of TCE, PCE and 1,4-dioxane between three contaminant degradation phase cycles. Rate of degradation during 2nd and 3rd cycles is calculated as % of rate of degradation during 1st cycle. (a) TCE, T1 = 1.1 $\mu\text{M h}^{-1}$, TP1 = 0.8 $\mu\text{M h}^{-1}$, TD1 = 0.9 $\mu\text{M h}^{-1}$, TPD1 = 0.8 $\mu\text{M h}^{-1}$; (b) PCE, P1 = 1.4 $\mu\text{M h}^{-1}$, PT1 = 1.6 $\mu\text{M h}^{-1}$, PD1 = 1.1 $\mu\text{M h}^{-1}$, TPD1 = 0.9 $\mu\text{M h}^{-1}$; (c) 1,4-Dioxane, D1 = 30.8 $\mu\text{M h}^{-1}$, DT1 = 10.9 $\mu\text{M h}^{-1}$, DP1 = 12.1 $\mu\text{M h}^{-1}$, TPD1 = 16.8 $\mu\text{M h}^{-1}$. Error bars represent range of errors in duplicate batch reactors.

Furthermore, lactate was only depleted to approximately 15 - 20 mM levels after three successive cycles (i.e., 30 - 40% of the initial 50 mM lactate feed remained after the third cycle), and correspondingly, acetate was produced (from lactate oxidation) at similar rates in each of the three cycles (Figs. S6-13). These results indicate that the microbially-driven Fenton reaction was not limited by lactate depletion upon completion of the third cycle, and that the microbially-driven Fenton system can handle at least three repeated feeds of ternary mixtures of TCE, PCE, and 1,4-dioxane without compromising the efficiency of contaminant degradation.

Rates of contaminant degradation by $\text{HO}^\bullet$ radicals are a function of the contaminant-specific $\text{HO}^\bullet$ radical reaction rate constants ($k_{\text{HO}^\bullet}$) (TCE range: $0.85\text{--}1.59 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, TCE average: $1.22 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$; PCE range: $1.62\text{--}2.33 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, PCE average: $1.98 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$; 1,4-dioxane range: $1.1\text{--}2.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, 1,4-dioxane average: $1.75 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) (25, 54), the initial concentrations of contaminant, and the number of double bonds in the contaminant molecular structure (26). Other factors impacting the Fenton degradation of TCE, PCE, and 1,4-dioxane include the myriad of unidentified $\text{HO}^\bullet$ radical-interacting intermediates produced during contaminant degradation (12, 38).

Despite these complicating factors, the predicted- and experimentally-derived contaminant degradation rates in the present study may be compared for the case of binary mixtures of TCE and PCE because both contaminants were added at identical concentrations and both contain an identical number of double bonds. Subsequently, the ratio of the experimentally-derived rates of PCE and TCE degradation in binary mixtures of PCE and TCE (designated $P^{\text{exp}}/T^{\text{exp}}$) is predicted to be equal to the ratio of PCE- and TCE-specific $k_{\text{HO}^\bullet}$ (designated $k_{\text{HO}^\bullet}(\text{P})/k_{\text{HO}^\bullet}(\text{T})$). $P^{\text{exp}}/T^{\text{exp}}$ ratios ranged from 1.8 - 2.1 in the three degradation phases of the PCE and TCE binary mixture experiments (Fig. 3), which were comparable to $k_{\text{HO}^\bullet}(\text{P})/k_{\text{HO}^\bullet}(\text{T})$ ratios (1.5 - 1.9) previously reported for TCE and PCE degradation by purely chemical Fenton reactions (indicated by the dotted lines in Fig. 3) (25).

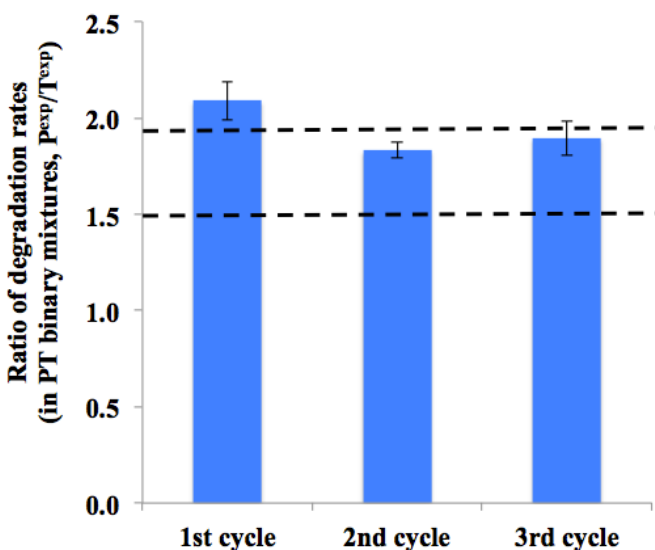


Figure 3. Ratio of the experimentally-derived rates of PCE and TCE degradation in binary mixtures of PCE and TCE (P^{exp}/T^{exp}) across three contaminant degradation phase cycles. Black dotted lines indicate reported range of ratio of $k_{HO^\bullet}(P)/k_{HO^\bullet}(T)$ (25). Equal molar of TCE and PCE (100 μ M) were added at the start of each cycle. Error bars represent range of errors in duplicate batch reactors.

Similar rate comparisons with binary mixtures of 1,4-dioxane and TCE or 1,4-dioxane and PCE were not possible because the source zone levels of 1,4-dioxane (2 mM) used in the present study were 20-fold greater than the source zone levels of TCE and PCE (100 μ M) (55, 56). Correspondingly, the 20-fold higher 1,4-dioxane concentrations led to 1,4-dioxane degradation rates that were 9-to-34-fold higher than the TCE and PCE degradation rates in all contaminant mixtures (Tables S6 and S7). Regardless of the concentration-dependent differences in contaminant degradation rates, the microbially-driven Fenton reaction degraded source zone levels of ternary mixtures of TCE, PCE, and 1,4-dioxane to below detection limits when contaminant degradation phases were extended to < 5-d time periods (first and second cycles; Figs. 1b, 1d, and 1f).

Rates of degradation of TCE as sole contaminant (in first, second, and third TCE degradation phases) were 18-54% lower in binary and ternary mixtures of PCE and 1,4-dioxane (i.e., by comparing rates of TCE degradation in phases T1, TP1, TD1, TPD1 and T2, TP2, TD2, TPD2 and T3, TP3, TD3, TPD3; Fig. 4a and Table S3). Decreases in TCE degradation rates in the binary and ternary contaminant mixtures are most likely due to competition for $HO^\bullet$ radicals by PCE (with a $k_{HO^\bullet}$ 62% higher than TCE) or 1,4-dioxane (with a $k_{HO^\bullet}$ approximately 43% higher than TCE and amended at 20-fold higher concentrations than TCE). Rates of degradation of PCE as sole contaminant (in first, second, and third PCE degradation phases) were not affected by TCE in binary mixtures of TCE and PCE, but were 4-38 % lower in binary and ternary mixtures containing 1,4-dioxane (i.e., by comparing rates of PCE degradation in phases P1, TP1, PD1, TPD1, and P2, TP2, PD2, TPD2 and P3, TP3, PD3, TPD3; Fig 4b and Table S4). The inability of TCE to affect PCE degradation rates is most likely due to the 62% higher $k_{HO^\bullet}$ for PCE than TCE. Decreases in PCE degradation rates in the binary and ternary contaminant mixtures containing 1,4-dioxane are most likely due to competition for $HO^\bullet$ radicals by 1,4-dioxane, which was amended at 20-fold higher concentrations than PCE. Rates of degradation of 1,4-dioxane as sole contaminant (in first, second, and third TCE degradation phases) were 39-65% lower in binary and ternary mixtures of TCE and PCE (i.e., by comparing rates of 1,4-dioxane degradation in phases

D1, TD1, PD1, TPD1 and D2, TD2, PD2, TPD2 and T3, TD3, PD3, TPD3; Fig 4c and Table S5). Decreases in 1,4-dioxane degradation rates in the binary and ternary contaminant mixtures are most likely due to competition for HO[•] radicals by PCE (with a $k_{\text{HO}^\bullet}$ 13% higher than 1,4-dioxane; Fig. 4c). During the third 1,4-dioxane degradation cycle, however, 1,4-dioxane degradation rates rebounded to 70 – 101% of the degradation rates with 1,4-dioxane as sole contaminant, most likely due to decreases in PCE concentrations below threshold levels for PCE to compete effectively with 1,4-dioxane for HO[•] radicals during the third contaminant degradation cycle (Fig. 4c).

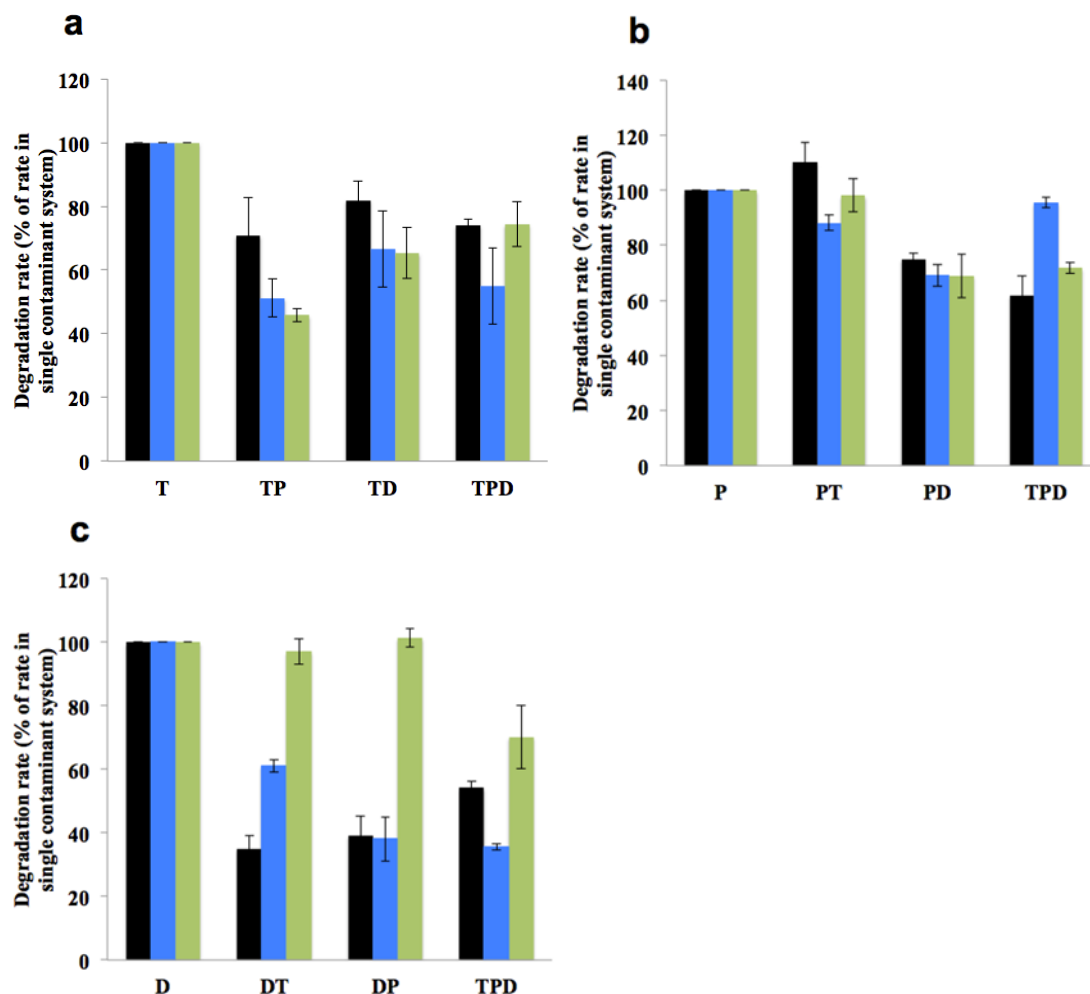


Figure 4. Comparison of rate of degradation of TCE, PCE and 1,4-dioxane between single, binary and ternary mixtures. Rate of degradation for binary and ternary mixtures is calculated as % of rate of single contaminant degradation. T1 = 1.1 $\mu\text{M h}^{-1}$, T2 = 1.5 $\mu\text{M h}^{-1}$, T3 = 1.4 $\mu\text{M h}^{-1}$, P1 = 1.4 $\mu\text{M h}^{-1}$, P2 = 1.6 $\mu\text{M h}^{-1}$, P3 = 1.2 $\mu\text{M h}^{-1}$, D1 = 30.8 $\mu\text{M h}^{-1}$, D2 = 36.2 $\mu\text{M h}^{-1}$, D3 = 27.9 $\mu\text{M h}^{-1}$. black, 1st cycle; blue, 2nd cycle; green, 3rd cycle. Error bars represent range of errors in duplicate batch reactors.

Similar to previous results reported with purely chemical Fenton reactions, results of the present study indicate that contaminant degradation rates in the microbially-driven Fenton reaction depend on the $k_{\text{HO}^\bullet}$ of competing contaminants, the initial contaminant concentration, and the number of double bonds in the contaminant molecular structure. In binary and ternary mixtures, contaminants with greater $k_{\text{HO}^\bullet}$ suppress the degradation of other contaminants with lower $k_{\text{HO}^\bullet}$ and the contaminant degradation phase must be extended for longer time periods to degrade

contaminants below detection limits (26). The microbially-driven Fenton reaction may thus be applied as an effective *ex situ* platform for simultaneous degradation of at least three (and potentially more) co-mingled contaminants at source zone levels.

2E. Conclusions and Implications for Future Research/ Implementation. Microbial respiratory processes catalyze both H₂O₂ production and Fe(III) re-reduction in the microbially-driven Fenton reaction designed in the present study. Microbial Fe(III) reduction is a dominant anaerobic respiratory process in many contaminated subsurface environments (57), which suggests that the microbially-driven Fenton reaction may be stimulated *in situ* by exposing Fe(III)-reducing facultative anaerobes in Fe(III)-containing contaminated environments to alternating anaerobic and aerobic conditions. Other *in situ* remediation strategies may include stimulation of the microbially-driven Fenton reaction by placing reactive iron barriers in the flow path of contaminated subsurface aquifers and exposing Fe(III)-reducing bacteria attached to the iron barriers to alternating anaerobic and aerobic conditions (58). In principal, targets for *ex situ* and *in situ* degradation by the microbially-driven Fenton reaction developed in the present study include multiple combinations of environmental contaminants susceptible to attack by Fenton reaction-generated HO[•] radicals, including co-mingled plumes of 1,4-dioxane, PCP (35, 36), PCE (37), TCE (23), TCA (59) and PFAS (60).

2F. Literature Cited.

1. Teel AL, Warberg CR, Atkinson DA, Watts RJ. 2001. Comparison of mineral and soluble iron Fenton's catalysts for the treatment of trichloroethylene. *Water Res* 35:977-984.
2. Wu X, Gu X, Lu S, Qiu Z, Sui Q, Zang X, Miao Z, Xu M, Danish M. 2015. Accelerated degradation of tetrachloroethylene by Fe(II) activated persulfate process with hydroxylamine for enhancing Fe(II) regeneration *Journal of Chemical Technology and Biotechnology* doi:10.1002/jctb.4718.
3. Chawla RC, Doura KF, McKay D. 2001. Effect of Alcohol Cosolvents on the Aqueous Solubility of Trichloroethylene. *Proceedings of the 2001 Conference on Environmental Research*:52-66.
4. Tsai TT, Kao CM, Hong A. 2009. Treatment of tetrachloroethylene-contaminated groundwater by surfactant-enhanced persulfate/BOF slag oxidation--a laboratory feasibility study. *J Hazard Mater* 171:571-576.
5. Choi K, Lee W. 2012. Enhanced degradation of trichloroethylene in nano-scale zero-valent iron Fenton system with Cu(II). *J Hazard Mater* 211-212:146-153.
6. Kang JW, Khan Z, Doty SL. 2012. Biodegradation of trichloroethylene by an endophyte of hybrid poplar. *Appl Environ Microbiol* 78:3504-3507.
7. Lanigan RS. 2000. Addendum to the final report on the safety assessment of polysorbates. *Int J Toxicol* 19:43-89.
8. Zenker MJ, Borden RC, Barlaz MA. 2003. Occurrence and treatment of 1,4-dioxane in aqueous environments. *Environ Eng Sci* 20:423-432.
9. Sei K, Kakinoki T, Inoue D, Soda S, Fujita M, Ike M. 2010. Evaluation of the biodegradation potential of 1,4-dioxane in river, soil and activated sludge samples. *Biodegradation* 21:585-591.
10. Abe A. 1999. Distribution of 1,4-dioxane in relation to possible sources in the water environment. *Sci Total Environ* 227:41-47.

11. Jackson RE, Dwarakanath V. 1999. Chlorinated degreasing solvents: Physical-chemical properties affecting aquifer contamination and remediation. *Ground Water Monitoring and Remediation* 19:102-110.
12. Stefan MI, Bolton JR. 1998. Mechanism of the degradation of 1,4-dioxane in dilute aqueous solution using the UV hydrogen peroxide process. *Environmental Science & Technology* 32:1588-1595.
13. Sun BZ, Ko K, Ramsay JA. 2011. Biodegradation of 1,4-dioxane by a *Flavobacterium*. *Biodegradation* 22:651-659.
14. Mahendra S, Alvarez-Cohen L. 2006. Kinetics of 1,4-dioxane biodegradation by monooxygenase-expressing bacteria. *Environmental Science & Technology* 40:5435-5442.
15. Kim YM, Jeon JR, Murugesan K, Kim EJ, Chang YS. 2009. Biodegradation of 1,4-dioxane and transformation of related cyclic compounds by a newly isolated *Mycobacterium* sp. PH-06. *Biodegradation* 20:511-519.
16. Vescovi T, Coleman HM, Amal R. 2010. The effect of pH on UV-based advanced oxidation technologies--1,4-dioxane degradation. *Journal of hazardous materials* 182:75-79.
17. Coleman HM, Vimonses V, Leslie G, Amal R. 2007. Degradation of 1,4-dioxane in water using TiO₂ based photocatalytic and H₂O₂/UV processes. *Journal of Hazardous Materials* 146:496-501.
18. Anderson RH, Anderson JK, Bower PA. 2012. Co-occurrence of 1,4-dioxane with trichloroethylene in chlorinated solvent groundwater plumes at US Air Force installations: Fact or fiction. *Integrated Environmental Assessment and Management* 8:731-737.
19. Stickney JA, Sager SL, Clarkson JR, Smith LA, Locey BJ, Bock MJ, Hartung R, Olp SF. 2003. An updated evaluation of the carcinogenic potential of 1,4-dioxane. *Reg Tox Pharm* 38:183-195.
20. USEPA. 2014. Technical Fact Sheet - 1,4-Dioxane. Response OoSWaE,
21. Cheng D, He J. 2009. Isolation and characterization of "Dehalococcoides" sp. strain MB, which dechlorinates tetrachloroethene to trans-1,2-dichloroethene. *Appl Environ Microbiol* 75:5910-5918.
22. Liang SH, Wang SY, Chang YM, Kao CM. 2015. Treatment of TCE-contaminated Groundwater using In Situ Potassium Permanganate Oxidation: Effects and Kinetics Evaluation. *Research Journal of Biotechnology* 10:20-24.
23. Tsai TT, Kao CM, Surampalli RY, Weng CH, Liang SH. 2010. Treatment of TCE-Contaminated Groundwater Using Fenton-Like Oxidation Activated with Basic Oxygen Furnace Slag. *Journal of Environmental Engineering-Asce* 136:288-294.
24. Jho EH, Singhal N, Turner S. 2010. Fenton degradation of tetrachloroethene and hexachloroethane in Fe(II) catalyzed systems. *J Hazard Mater* 184:234-240.
25. Yeh CK, Hsu CY, Chiu CH, Huang KL. 2008. Reaction efficiencies and rate constants for the goethite-catalyzed Fenton-like reaction of NAPL-form aromatic hydrocarbons and chloroethylenes. *J Hazard Mater* 151:562-569.
26. Yeh KJ, Chen TC, Young WL. 2013. Competitive Removal of Two Contaminants in a Goethite-Catalyzed Fenton Process at Neutral pH. *Environmental Engineering Science* 30:47-52.
27. Rashid MM, Sato C. 2012. Degradation of Trichloroethylene and Tetrachloroethylene in Simulated Groundwater in a Flow-Through Photosono Reactor. *Journal of Environmental Engineering-Asce* 138:1179-1185.

28. Pavlostathis SP, Prytula MT, Yeh DH. 2003. Potential and limitations of microbial reductive dechlorination for bioremediation applications. *Water, Air and Soil Pollution: Focus* 3:117-129.
29. Sekar R, DiChristina TJ. 2014. Microbially driven fenton reaction for degradation of the widespread environmental contaminant 1,4-dioxane. *Environ Sci Technol* 48:12858-12867.
30. Klecka GM, Gonsior SJ. 1986. Removal of 1,4-Dioxane from wastewater. *Journal of Hazardous Materials* 13:161-168.
31. Hill RR, Jeffs GE, Roberts DR. 1997. Photocatalytic degradation of 1,4-dioxane in aqueous solution. *J Photochem Photobiol A* 108:55-58.
32. Beckett MA, Hua I. 2003. Enhanced sonochemical decomposition of 1,4-dioxane by ferrous iron. *Water Research* 37:2372-2376.
33. Che H, Bae S, Lee W. 2011. Degradation of trichloroethylene by Fenton reaction in pyrite suspension. *J Hazard Mater* 185:1355-1361.
34. Tyre BW, Watts RJ, Miller GC. 1991. Treatment of 4 Biorefractory Contaminants in Soils Using Catalyzed Hydrogen-Peroxide. *Journal of Environmental Quality* 20:832-838.
35. Barbeni M, Minero C, Pelizzetti E, Borgarello E, Serpone N. 1987. Chemical Degradation of Chlorophenols with Fenton Reagent ($\text{Fe}^{2+} + \text{H}_2\text{O}_2$). *Chemosphere* 16:2225-2237.
36. McKinzi AM, Dichristina TJ. 1999. Microbially driven Fenton reaction for transformation of pentachlorophenol. *Environmental Science & Technology* 33:1886-1891.
37. Jho EH, Singhal N, Turner S. 2010. Fenton degradation of tetrachloroethene and hexachloroethane in $\text{Fe}(\text{II})$ catalyzed systems. *Journal of hazardous materials* 184:234-240.
38. Pignatello J, Liu D, Huston P. 1999. Evidence for an Additional Oxidant in the Photoassisted Fenton Reaction. *Environmental Science & Technology* 33:1832-1839.
39. Ojinnaka C, Osuji L, Achugasim O. 2012. Remediation of hydrocarbons in crude oil-contaminated soils using Fenton's reagent. *Environ Monit Assess* 184:6527-6540.
40. Lewis S, Lynch A, Bachas L, Hampson S, Ormsbee L, Bhattacharyya D. 2009. Chelate-Modified Fenton Reaction for the Degradation of Trichloroethylene in Aqueous and Two-Phase Systems. *Environ Eng Sci* 26:849-859.
41. Kim CG, Seo HJ, Lee BR. 2006. Decomposition of 1,4-dioxane by advanced oxidation and biochemical process. *J Environ Sci Health A Tox Hazard Subst Environ Eng* 41:599-611.
42. Hand S, Wang B, Chu KH. 2015. Biodegradation of 1,4-dioxane: effects of enzyme inducers and trichloroethylene. *Sci Total Environ* 520:154-159.
43. Shim H, Ryoo D, Barbieri P, Wood TK. 2001. Aerobic degradation of mixtures of tetrachloroethylene, trichloroethylene, dichloroethylenes, and vinyl chloride by toluene-o-xylene monooxygenase of *Pseudomonas stutzeri* OX1. *Appl Microbiol Biotechnol* 56:265-269.
44. Marco-Urrea E, Gabarrell X, Caminal G, Vicent T, Reddy CA. 2008. Aerobic degradation by white-rot fungi of trichloroethylene (TCE) and mixtures of TCE and perchloroethylene (PCE). *Journal of Chemical Technology and Biotechnology* 83:1190-1196.
45. Sambrook J, Fritsh EF, Maniatis T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY.
46. Obuekwe CO, Westlake DWS, Cook FD. 1981. Effect of Nitrate on Reduction of Ferric Iron by a Bacterium Isolated from Crude-Oil. *Canadian Journal of Microbiology* 27:692-697.
47. Dichristina TJ, Delong EF. 1994. Isolation of Anaerobic Respiratory Mutants of *Shewanella-Putrefaciens* and Genetic-Analysis of Mutants Deficient in Anaerobic Growth on Fe^{3+} . *Journal of Bacteriology* 176:1468-1474.

48. Howard PH. 1990. Handbook of Environmental Fate and Exposure Data for Organic Chemicals. Lewis Publishers, Inc, Chelsea, MI.
49. ATSDR. 1993. Agency for Toxic Substances and Disease Registry. Toxicological Profile for Tetrachloroethylene. Update. CDC, Atlanta, GA.
50. Boulding RJ. 1996. EPA Environmental Engineering Sourcebook. Ann Arbor Press, Chelsea, MI.
51. P.J. Linstrom PJ, Mallard WG. NIST Chemistry WebBook, NIST Standard Reference Database Number 69, vol 20899. National Institute of Standards and Technology, Gaithersburg MD.
52. Dichristina TJ. 1992. Effects of Nitrate and Nitrite on Dissimilatory Iron Reduction by *Shewanella-Putrefaciens*-200. *Journal of Bacteriology* 174:1891-1896.
53. Andreozzi R, Campanella L, Frayse B, Garric J, Gonnella A, Giudice RL, Marotta R, Pinto G, Pollio A. 2004. Effects of advanced oxidation processes (AOPs) on the toxicity of a mixture of pharmaceuticals. *Water Sci Technol* 50:23-28.
54. Adams CD, Scanlan PA, Secrist ND. 1994. Oxidation and biodegradability enhancement of 1,4-dioxane using hydrogen peroxide and ozone. *Environ Sci Technol* 28:1812-1818.
55. Steffan RJ. 2007. Biodegradation of 1,4-Dioxane.
56. Farm R. 2001. Public Health Assessment. The New Jersey Department of Health and Senior Services, Dover Township, Ocean County, New Jersey.
57. Lovley DR. 1997. Microbial Fe(III) reduction in subsurface environments. *FEMS Microbiology Reviews* 20:305-313.
58. Cundya AB, Hopkinsona L, Whitby RLD. 2008. Use of iron-based technologies in contaminated land and groundwater remediation: A review. *Science of the Total Environment* 400:42-51.
59. Joseph J. Pignatello, Di Liu, Huston. P. 1999. Evidence for an Additional Oxidant in the Photoassisted Fenton Reaction. *Environmental Science & Technology* 33:1832-1839.
60. Cao MH, Wang BB, Yu HS, Wang LL, Yuan SH, Chen J. 2010. Photochemical decomposition of perfluorooctanoic acid in aqueous periodate with VUV and UV light irradiation. *J Hazard Mater* 179:1143-1146.
61. Sekar RS, DiChristina TD. 2016. Simultaneous Degradation of Comingled Contaminants Trichloroethylene, Tetrachloroethylene, and 1,4-Dioxane by a Microbially Driven Fenton Reaction. *Environmental Microbiology* 82(21):6335-6343.

3. Application of the Microbially-Driven Fenton Reaction for Degradation of the Perfluoroalkyl Substance Perfluorooctanoic Acid (PFOA)

3A. Objective. The objective of this research was to develop novel remediation technologies using the microbially-driven Fenton reaction for degradation of PFOA.

3B. Background. Perfluoroalkyl substances (PFAS) have received recent heightened attention as emerging contaminants and persistent organic pollutants by global governmental agencies, including the USEPA. Due to their widespread application in household products and as the active component of aqueous film-forming foams, PFAS are globally distributed in the environment with significant concentrations detected in drinking water, lakes, and even in remote locations such as the Arctic Ocean (Hu 2016; Moller 2010; Gonzalez-Gaya 2014, Kannan et al 2004, Yeung 2017). PFAS contain polar head groups attached to fluorinated carbon chains, which resist degradation due to the high strength of the carbon-fluorine bond (116 kcal/mol) (O'Hagan 2008) (Fig. 1). PFAS thus bioaccumulate in the blood and tissues of marine animals, land mammals and humans and can display carcinogenic properties (Moller 2010; Casal 2017; Bjerregaard-Olesen 2015).

Some effective PFAS remediation techniques such as sonolysis or plasma treatment are often resource or energy intensive, but advanced oxidation processes (AOP) or reduction strategies may be a viable alternative for *in-situ* treatment of contaminated water (Moriwaki 2005; Vecitis 2008; Wu 2019; Stratton 2017). Prior to 2020, there was considerable ambiguity about whether the HO• radical, utilized in some AOPs, can effectively attack and degrade PFAS, including PFOA. Reports indicated PFOA and other PFAS were degraded by a UV-Fenton reaction (Tang 2012), photodegradation in a UV- Fe(III) system (Liu 2013), catalyzed hydrogen peroxide propagation with hydroxyl radical and superoxide (Mitchell 2014), and solvated electrons (Park 2009). Additionally, if not directly involved, there was evidence that superoxide or the hydroxyl radical may assist PFOA degradation (Chen 2016, 2015, 2011; Gomez-Ruiz 2018; Huang 2016; Lin 2015). PFAS decontamination via microbial (enzymatic) PFAS defluorination has yet to be definitively demonstrated, most likely due to the strength of the carbon-fluorine bond and resistance to enzymatic attack.

The microbially driven Fenton reaction produces the hydroxyl radical (HO•), which indiscriminately attacks and degrades contaminants, and thus is distinct from other microbial degradation techniques which typically require expression of a specific enzyme to degrade a single contaminant. The microbially driven Fenton reaction produces hydroxyl radicals that have degraded a wide range of environmental contaminants including pentachlorophenol; 1,4-dioxane; trichloroethylene; perchloroethylene; pyrene; and anthracene (McKinzi 1999; Sekar 2014; Sekar 2016; Sekar 2017). In contrast to conventional (purely abiotic) Fenton remediation, microbially driven Fenton operates at a circumneutral pH, does not require exogenous addition of reagents, and regenerates catalysts. We applied the microbially driven Fenton reaction system to the emerging contaminant PFOA. Bioreactors contained M1 minimal growth medium amended with Fe(III)-citrate, PFOA, and the facultative anaerobe *Shewanella oneidensis* MR-1. The bioreactor was initially degassed to allow ferric iron respiration by *S. oneidensis* MR-1, which reduce all available Fe(III) to Fe(II). After all iron was reduced, the reactor was sparged with compressed air and cells switched to aerobic respiration, which produces hydrogen peroxide (H₂O₂) and superoxide as waste products. Ferrous iron (Fe(II)) and H₂O₂ react to produce the hydroxyl radical, which can initiate a chain of radical reactions to attack and degrade the environmental pollutants.

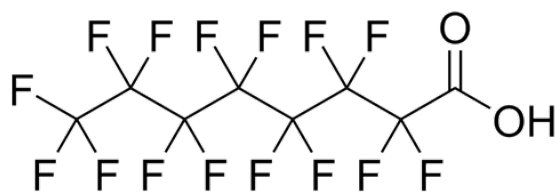


Figure 1. Structure of environmental pollutant perfluorooctanoic acid.

3C. Materials and Methods.

Design of a Microbially Driven Fenton Reaction for Perfluorooctanoic Acid in a Batch Reactor System. The toxicity of PFOA to *S. oneidensis* was tested by growing batch cell cultures at cell density $OD_{600}=0.1$ in the presence of high concentrations (0-300 μM) of PFOA (Sigma-Aldrich) in M1 minimal medium under anaerobic conditions with 5mM Fe(III) citrate for 48 h. Cell activity was monitored by measuring the concentration of Fe(III) reduced to Fe(II) over time using the ferrozine method (Stookey 1970). The experimental conditions for the microbially-driven Fenton degradation of PFOA were nearly identical to the batch system designed to degrade 1,4-dioxane in (Sekar 2014). Each bioreactor contained *S. oneidensis* batch cultures at $OD_{600} = 1.0$ amended with 20 mM lactate as the electron donor, 10 mM Fe(III)-citrate as the electron acceptor, and 1 μM PFOA, and exposed to an aerobic/anaerobic cycling period of 2-4 hours. Reactor temperature (25°C) and pH (7.0) were held constant in all experiments. Aerobic conditions were initiated by sparging the culture with hydrated compressed air for pre-selected time periods, and anaerobic conditions were initiated and maintained in a similar manner with N_2 gas. Each bioreactor had two experimental replicates.

Liquid-Liquid Extraction of PFOA from Batch Reactors. Although there are numerous PFOA extraction protocols found in the literature, most are optimized for extraction from comparatively dilute or low-concentration environments: drinking water, marine waters, or groundwater, where PFOA concentrations are generally in the nano- or picomolar range, and sample contamination with the analyte is a concern. In contrast, our bioreactors presented a high concentration of PFOA at 1 μM , high iron and iron oxide mineral content (10 mM Fe(III)), and high cell density (10^9 cells $\cdot \text{mL}^{-1}$). As such, we did not need to take precaution against trace contamination with the analyte and prioritized maximizing extraction recovery yield. Immediately following batch reactor decommission, 2 mL of liquid culture samples were aliquoted into microcentrifuge tubes. Each sample was spiked with isotopically labeled M2PFOA (extraction control; Wellington Laboratories, Inc.) (Fig. 2) for a final concentration of 100nM and stored at 4C. 3 mL of HPLC-grade water (pH=10, adjusted with NaOH) was added to 0.5 mL of liquid culture in a 15 mL borosilicate glass tube, then vortexed on high speed for 10 seconds and incubated at room temperature for 30 minutes. 4 mL of methyl tert-butyl ether (MTBE; Sigma-Alrich) was added to the tube and vortexed on high for 20 minutes. Tubes were set upright to allow separation of aqueous and organic layers, then MTBE (organic layer containing PFOA) was removed using a glass syringe with a steel luer-lock needle to a new 15mL borosilicate glass tube (all glass and steel materials were required for extractions due to incompatibility of MTBE with most plastic materials). MTBE extraction steps were repeated three times, then stored at 4C if required. Samples were centrifuged in a Speedvac at 4000rpm for 1.5 hrs to evaporate MTBE from tubes, then analyte was resuspended in 1mL of 90:10 H_2O :acetonitrile. Each sample was spiked with isotopically labeled M8PFOA (instrument control; Wellington Laboratories, Inc.) (Fig. 2) at a final concentration of 100 nM. Samples were vortexed and transferred to HPLC vials, then analyzed using liquid chromatography combined with tandem mass spectrometry (LC-MS/MS).

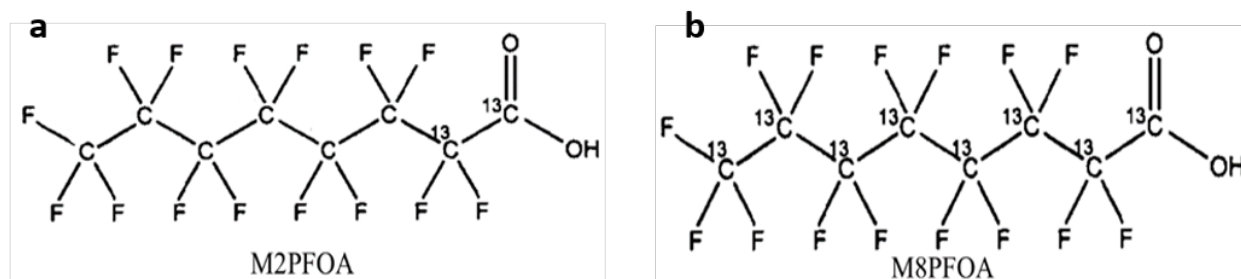


Figure 2. Structures of ^{13}C -labeled PFOA standards. a) M2PFOA, double- ^{13}C -labeled extraction control; b) M8PFOA, octuple- ^{13}C -labeled instrument control.

Quantification of PFOA using Tandem Mass Spectrometry Combined with Liquid Chromatography. LC-MS/MS method development for PFOA detection was completed using the Agilent 1100 Series HPLC interfaced with a Micromass Quattro LC triple quadrupole mass spectrometer. All data shown is an average from three technical replicate injections. In every run, blank samples were injected first to eliminate/reduce sample carryover, low concentration neat samples (namely control M2FOA and M8PFOA) were injected next for the same reason, then followed by experimental samples. The $1\ \mu\text{M}$ PFOA neat control was injected last. The HPLC was operated using water and acetonitrile (ACN) both containing 5mM ammonium acetate and a $50 \times 2 \times 10\ \text{mm}$ Kinetex C18 column (mm length $\times$ mm inside diameter $\times$ μm particle size) kept at 25°C . Pumping at a constant total flow rate of $0.5\text{mL}/\text{min}$, runs begin with an eluent of 90/10 (v/v) water/ACN, then linearly ramping to 30/70 at 2 min, changing to 0/100 at 9 min, holding constant until 11 min, linearly ramping to 5/95 until 12 min, then holding constant at 5/95 until end of analysis at 15 min. PFOA elutes around 7.5 min. Upon elution from the HPLC, extracts are introduced to the mass spectrometer in ESI(-) mode with the capillary potential set at $-3.5\ \text{kV}$, the cone potential at -20V , the extractor potential at $-8\ \text{V}$ and the radio frequency (rf) lens potential at $0.05\ \text{V}$. The source block temperature was maintained at 100°C and the desolvation gas temperature was maintained at 350°C . Both the low and high mass resolutions in the first quadrupole were set to 14.0 (unitless ratio of direct to rf current voltages) and the ion energy was set to $0.6\ \text{V}$. In the collision cell, the entrance and exits were set to 25V . The low and high mass resolutions were set at 14.0 in the third quadrupole and the ion energy was set at $1.6\ \text{V}$. The detector multiplier was set to -650V .

3D. Results and Discussion. Toxicity of PFOA to *S. oneidensis* was negligible between $0\text{--}50\ \mu\text{M}$, with effects seen between $100\text{--}300\ \mu\text{M}$ (Fig. 3). Initially, microbially-driven Fenton bioreactor experiments were amended with $10\ \mu\text{M}$ PFOA (data not shown), but eventually were switched to $1\ \mu\text{M}$ to better resolve any potential degradation activity.

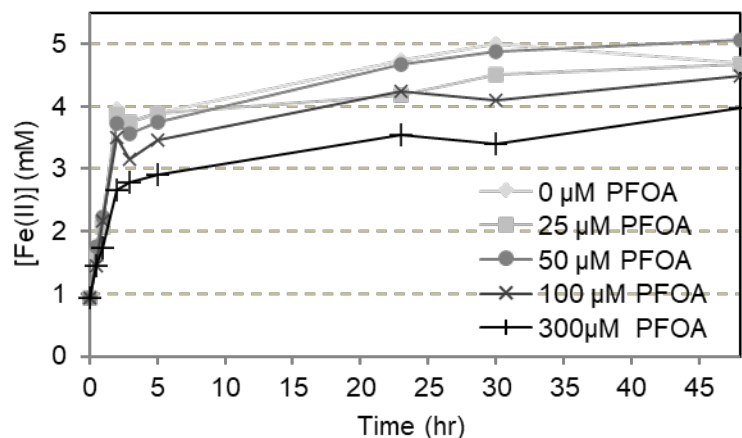


Figure 3. Toxicity of PFOA to *S. oneidensis* MR-1 incubated with 5 mM Fe(III)-citrate. Cells were incubated in anoxic batch reactors for 48 hours in M1 minimal medium amended with 20mM lactate, 5mM Fe(III)-citrate, and 0-300 μ M PFOA. Samples were taken periodically and analyzed for Fe(II) as a metric for tracking cell activity using the ferrozine method (Stookey 1970).

After four cycles of the microbially driven Fenton reaction, endpoint samples from each bioreactor were analyzed for remaining PFOA. Controls included bioreactors amended with hydroxyl radical scavengers mannitol (120 mM) and thiourea (40 mM), and bottles that lacked cells, Fe(III) or PFOA. All bioreactors reduced and reoxidized Fe(III)-citrate at similar rates as each other in all cycles (Fig. 4), indicating PFOA had no deleterious effect on *S. oneidensis*.

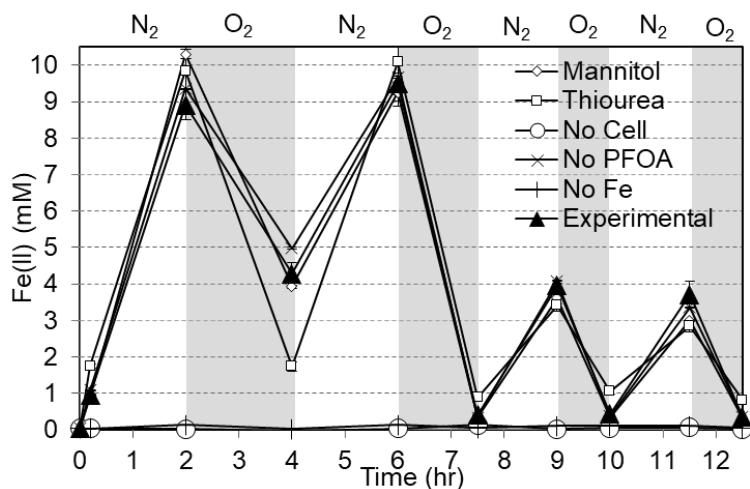


Figure 4. Fe(II) concentrations during four cycles of microbially driven Fenton bioreactors amended with 1 μ M PFOA. Error bars (standard deviation) are present but may be hidden behind markers. *S. oneidensis* batch cultures were incubated in M1 minimal medium at $OD_{600} = 1.0$ amended with Fe(III) and 1 μ M PFOA, and exposed to an aerobic/anaerobic cycling period of 2-4 hours. Reactor temperature (25°C) and pH (7.0) were held constant in all experiments. Aerobic conditions were initiated by sparging the culture with hydrated compressed air when Fe(II) was significantly oxidized, and anaerobic conditions were initiated and maintained with N_2 gas in a similar manner when Fe(III) was significantly reduced.

200 nM M2PFOA was spiked into samples immediately after bioreactors were decommissioned as an extraction control. M2PFOA percent recoveries were all similar to each other (no PFOA, 81%; experimental, 70%; abiotic, 75%; no Fe, 76%) (Fig. 5b), but skewed low when compared with the neat 200 nM M2PFOA control, indicating that some M2PFOA

(approximately 20% or 20 nM) likely remained in the aqueous layer after extraction. Increasing the M2PFOA concentration to 300 nM would likely resolve this issue. The M8PFOA instrument control worked well with all samples within 5% of the 100 nM neat sample signal intensity (no PFOA, 102%; experimental, 95%; abiotic, 97%; no Fe, 96%) (Fig. 5c).

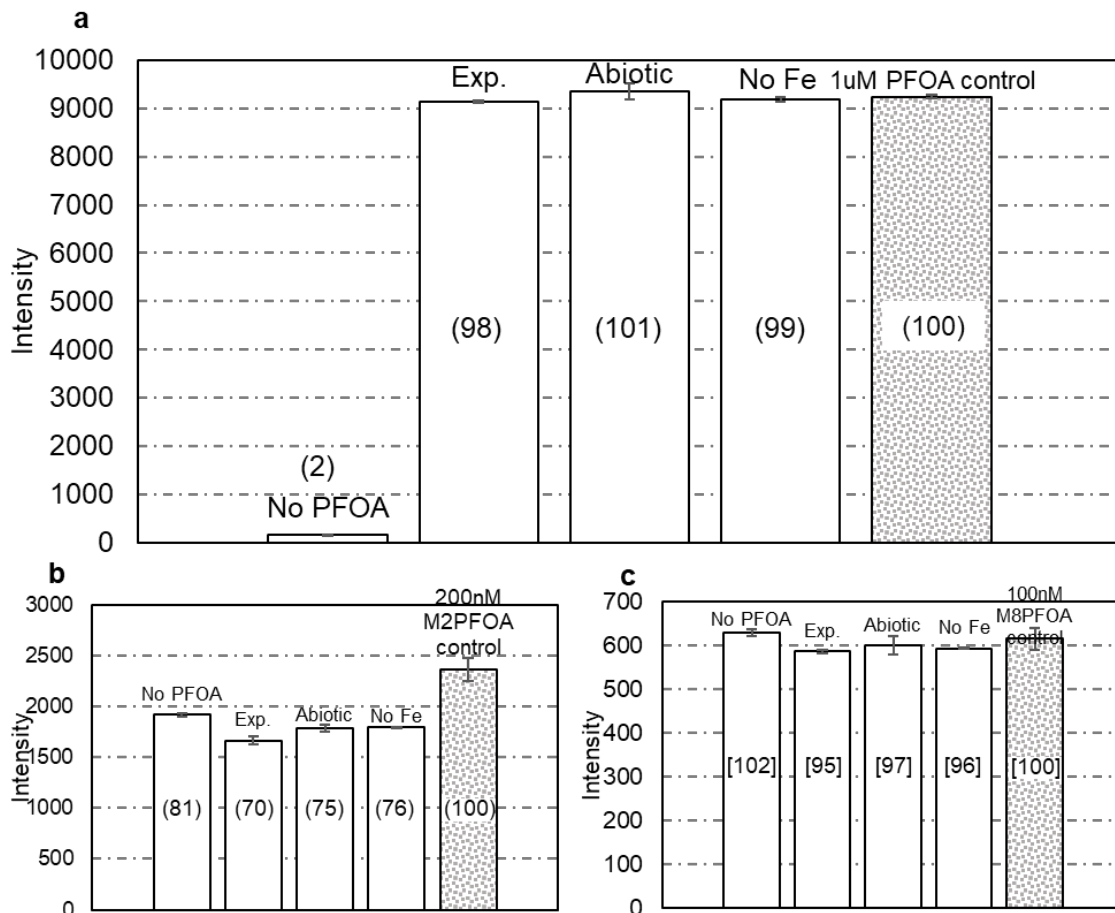


Figure 5. **a.** Intensity of residual PFOA in decommissioned bioreactors following MTBE extraction and LC-MS/MS quantitation (extraction percent recovery in parentheses, %). **b.** Intensity of residual M2PFOA (extraction control) in bioreactors after MTBE extraction and LC-MS/MS quantitation (extraction percent recovery in parentheses, %). **c.** Intensity of instrument control M8PFOA, which was spiked into each sample immediately following MTBE extraction (percentage of neat 100 nM control in [brackets], %).

PFOA extraction percent recoveries were excellent, recovering almost all of the original 1 μM $\pm$ 20 nM (Fig. 5a), indicating the PFOA extraction method is robust enough to recover all PFOA even under high iron, cell density, and metabolite conditions. However, this also indicates no PFOA was degraded by the microbially-driven Fenton reaction. No bioreactors showed evidence of any PFOA degradation after MTBE extraction and LC-MS/MS analysis (no PFOA, 2%; experimental, 98%; abiotic, 101%; no Fe, 99%) when compared with the neat 1 μM control sample (Fig. 5a). We attempted several iterations of bioreactors, changing variables including cell density, cycling times, Fe(III)-citrate concentration, and cell growth medium, but no PFOA was degraded (representative data in Fig. 5). Recent literature asserts that PFOA is not degraded by the hydroxyl radical (Javed 2020a) or superoxide (Javed 2020b), nor does the hydroxyl radical provide ancillary benefits to other radical attacks. Due to PFOA's recalcitrance to the microbially driven

Fenton reaction and the hydroxyl radical, it was not prudent to pursue degradation of other PFAS via the microbially driven Fenton reaction. However, there are other promising PFAS degradation mechanisms that are independent of the hydroxyl radical, including degradation by solvated electron attack (Park 2009) and zero-valent iron (Arvaniti 2015; Hori 2006).

3E. Conclusions and Future Implications. Due to PFOA's recalcitrance to the microbially driven Fenton reaction and the hydroxyl radical (JAVED 2020), we did not pursue degradation of PFOS or other PFAS.

3F. Literature Cited.

Arvaniti, OS, Hwang, Y, Andersen, HR, et al. Reductive degradation of perfluorinated compounds in water using Mg amino clay coated nanoscale zero valent iron. *Chem. Eng. J.* 2015; **262**: 133-139.

Bjerregaard-Olesen, C, Bossi R, Betch B, et al. Extraction of perfluorinated alkyl acids from human serum for determination of the combined xenoestrogenic transactivity: a method development. *Chemosphere* 2015; **129**: 232-8.

Casal P, Gonzalez-Gaya B, Zhang Y, et al. Accumulation of Perfluoroalkylated Substances in Oceanic Plankton. *Environ Sci Technol*, 2017. **51**(5): p. 2766-2775.

Chen MJ, Lo SL, Lee YC, et al. Photocatalytic decomposition of perfluorooctanoic acid by transition-metal modified titanium dioxide. *J. Hazard Mater.* 2015; **288**: 168-175.

Chen MJ, Lo SL, Lee YC, et al. Decomposition of perfluorooctanoic acid by ultraviolet light irradiation with Pb-modified titanium dioxide. *J. Hazard Mater.* 2016; **303**: 111-118.

Chen MJ, Lo SL, Kuo J. Effects of titanate nanotubes synthesized by a microwave hydrothermal method on photocatalytic decomposition of perfluorooctanoic acid. *Water Res.* 2011; **45**(14): 4131-4140.

Gomez-Ruiz B, Ribao P, Diban N, et al. Photocatalytic degradation and mineralization of perfluorooctanoic acid (PFOA) using a composite TiO₂-rGO catalyst. *J. Hazard Mater.* 2018; **344**: 950-957.

Gonzalez-Gaya B, Dachs J, Roscales J, et al. Perfluoroalkylated substances in the global tropical and subtropical surface oceans. *Environ Sci Technol*, 2014; **48**(22): p. 13076-84.

Hori H, Hayakawa E, Einaga H, et al. Decomposition of environmentally persistent perfluorooctanoic acid in water by photochemical approaches. *Environ Sci Technol*, 2004; **38**(22): 6118-24.

Hu XC, Andrews D, Lindstrom A, et al. Detection of Poly- and Perfluoroalkyl Substances (PFASs) in U.S. Drinking Water Linked to Industrial Sites, Military Fire Training Areas, and Wastewater Treatment Plants. *Environ Sci Technol Lett*, 2016; **3**(10): 344-350.

Huang J, Wang X, Pan Z, et al. Efficient degradation of perfluorooctanoic acid (PFOA) by photocatalytic ozonation. *Chem. Eng. J.* 2016; **296**: 329-334.

Javed H, Lyu C, Sun R, et al. Discerning the Inefficacy of Hydroxyl Radicals during Perfluorooctanoic Acid Degradation. *Chemosphere* 2020a, **247**: 125883.

Javed H, Metz J, Eraslan T, et al. Discerning the Relevance of Superoxide in PFOA Degradation
Cite This: *Environ. Sci. Technol. Lett.* 2020b; **7**: 653–658

Kannan K, Corsolini S, Falandysz J, et al. Perfluorooctanesulfonate and related fluorochemicals in human blood from several countries. *Environ Sci Technol*, 2004. **38**(17): 4489-95.

Lin JC, Lo SL, Hu CY, et al. Enhanced sonochemical degradation of perfluorooctanoic acid by sulfate ions. *Ultrason. Sonochem.* 2015; **22**: 542e547.

Liu D, Xiu Z, Liu F, et al. Perfluorooctanoic acid degradation in the presence of Fe(III) under natural sunlight. *J Hazard Mater*, 2013. **262**: 456-63.

McKinzi, A; DiChristina, TJ. Microbially Driven Fenton Reaction for Transformation of Pentachlorophenol. *Environmental Science and Technology* 1999; **33**(11) 1886-1891.

Mitchell, SM, Ahmad M, Teel AL, et al. Degradation of Perfluorooctanoic Acid by Reactive Species Generated through Catalyzed H₂O₂ Propagation Reactions. *Environmental Science & Technology Letters*, 2014; **1**(1): 117-121.

Moller A, Ahrens L, Surm R, et al. Distribution and sources of polyfluoroalkyl substances (PFAS) in the River Rhine watershed. *Environ Pollut*, 2010; **158**(10): 3243-50.

Moriwaki H, Takagi Y, Tanaka M, et al. Sonochemical Decomposition of Perfluorooctane Sulfonate and Perfluorooctanoic Acid. *Environ. Sci. Technol.* 2005; **39**: 3388– 3392.

O'Hagan D. Understanding organofluorine chemistry: An introduction to the C-F bond. *Chem Soc Rev*, 2008; **37**(2): 308-19.

Park H, Vecitis CD, Cheng J, et al. Reductive defluorination of aqueous perfluorinated alkyl surfactants: effects of ionic headgroup and chain length. *J. Phys. Chem.* 2009; **113**: 690-696.

Sekar RS, DiChristina TJ. Microbially Driven Fenton Reaction for Degradation of the Widespread Environmental Contaminant 1,4-Dioxane. *Environ. Sci. and Technol.* 2014; **48**: 12858-12867.

Sekar RJ, DiChristina TJ. Degradation of the recalcitrant oil spill components anthracene and pyrene by a microbially driven Fenton reaction. *FEMS Microbiol. Lett.* 2017; **364** (21).

Sekar RS, DiChristina TD. Simultaneous Degradation of Co-mingled Contaminants Trichloroethylene, Tetrachloroethylene, and 1,4-Dioxane by a Microbially Driven Fenton Reaction. *Environ. Microbiol.* 2017; **82**(21): 6335-6343.

Stookey LL. Ferrozine: a new spectrophotometric reagent for iron. *Anal. Chem.* 1970; 42: 779-781.

Stratton GR, Dai F, Bellona CL, et al. Plasma-Based Water Treatment: Efficient Transformation of Perfluoroalkyl Substances in Prepared Solutions and Contaminated Groundwater. *Environ. Sci. Technol.* 2017; **51**: 1643–1648.

Tang H, Xiang Q, Lei M, et al. Efficient degradation of perfluorooctanoic acid by UV-Fenton process. *Chem. Eng. J.* 2012; **184**: 156-162.

Vecitis CD, Park H, Cheng J, Mader BT, et al. Kinetics and Mechanism of the Sonolytic Conversion of the Aqueous Perfluorinated Surfactants, Perfluorooctanoate (PFOA), and Perfluorooctane Sulfonate (PFOS) into Inorganic Products. *J. Phys. Chem. A.* 2008, **112**: 4261–4270.

Wu B, Hao S, Choi Y, et al. Rapid Destruction and Defluorination of Perfluorooctanesulfonate by Alkaline Hydrothermal Reaction. *Environ. Sci. Technol. Lett.* 2019; **6**: 630–636.

Yeung LWY, Dassuncao C, Mabury S, et al. Vertical Profiles, Sources, and Transport of PFASs in the Arctic Ocean. *Environ Sci Technol*, 2017; **51**(12): 6735-6744

4. Optimizing the Microbially-Driven Fenton Reaction in Flow-Through Column Reactors

4A. Objective. As iron oxyhydroxides and iron-reducing bacteria are ubiquitous in soils and sediments, the microbially-driven Fenton reaction may represent an economical in situ bioremediation strategy for organic contaminants. The overall objectives of this study were to: (i) determine whether natural Fe(III) oxyhydroxide substrates promote the microbially-driven Fenton process; (ii) determine whether the microbially-driven Fenton reaction process and the degradation of 1,4-dioxane as example of contaminant can be sustained in flow-through columns (FTCs); and (iii) study the mechanism of the reaction and optimize experimental conditions to make the microbially-driven Fenton reaction sustainable in FTCs, with the ultimate objective of transferring this technology to pump-and-treat applications.

4B. Background. The microbially-driven Fenton reaction may represent an economical in situ bioremediation strategy for organic contaminants, as the Fenton degradation of organic contaminants may be fueled by facultative anaerobic microorganisms that may produce H_2O_2 and other reactive oxygen species (ROS) as by-product of aerobic respiration and Fe(II) from anaerobic respiration on a Fe(III) substrate [Sekar, R.; DiChristina, T. J., Sekar, R.; Taillefert, M.; DiChristina, T. J.]. Such approach requires alternating redox conditions between aerated and anaerobic conditions and has demonstrated efficient in batch reactors with ferric citrate as terminal electron acceptor in a minimum medium containing large concentrations of orthophosphate as buffer. To optimize the process for in situ bioremediation, it is necessary to determine whether the use of natural Fe(III) substrates as source of Fe(II) may represent a good alternative to soluble Fe(III) compounds. In addition, high concentrations of orthophosphate may inhibit Fe(III) oxyhydroxide dissolution [Stumm, W. Reactivity at the mineral-water interface: dissolution and inhibition.] and immobilize iron as phosphate minerals [Borch, T.; Fendorf, S. Phosphate Interactions with Iron (Hydr)oxides: Mineralization Pa, Zachara, J. M.; Kukkadapu, R. K.; Fredrickson, J. K.; Gorby, Y. A.; Smith, S. C. Bi], whereas low orthophosphate concentrations may impair microbial growth. Orthophosphate concentrations have therefore to be optimized to ensure enough bacterial growth while simultaneously promoting microbial reduction of the Fe(III) substrate and maintaining the Fe(II) by-product available for the Fenton reaction. In parallel, the microbial reduction of Fe(III) oxyhydroxides is often associated with the production of secondary mineralization by-products, including magnetite, goethite, siderite, and vivianite [Borch, T.; Fendorf, S. Phosphate Interactions with Iron (Hydr)oxides: Mineralization Pa-Hansel, C. M.; Benner, S. G.; Fendorf, S.]. The impact of these secondary minerals on the sustainability of the microbially-driven Fenton reaction remains unknown and has to be assessed. Finally, it is necessary to optimize the microbially-driven Fenton reaction process in flow-through column (FTC) experiments as a first step towards the development of a realistic bioremediation scheme that could be adapted for in situ bioremediation in future projects. FTC reactors are often used to pump-and-treat contaminated waters because they allow the treatment of a large volume of contaminated subsurface waters, they allow the recycling of essential components of the bioremediation process, and they can easily be manipulated to alter the biogeochemical conditions required to promote the degradation process by changing the pore water composition over time [Beazley, M. J.; Webb, S. M.; Martinez, R. J.; Sobecky, P. A.; Taillefert, M., Carey, E. A.;

Taillefert, M.]. In this particular case, the redox oscillations needed to promote in situ H_2O_2 and Fe(II) formation may be easily adopted in such reactor configuration.

The overall objectives of this part of the project were to: (i) determine whether ferrihydrite promotes the microbially-driven Fenton process; (ii) determine whether redox conditions in FTCs have to be alternated between aerobic conditions to promote the generation of ROS and anaerobic conditions to promote Fe(III) reduction as in the batch reactor experiments [Sekar, R.; DiChristina, T. J. ,Sekar, R.; Taillefert, M.; DiChristina, T. J.]; (iii) optimize the experimental conditions to maximize production of ROS and production of Fe(II) ; and (iv) determine whether microbially-driven Fenton reaction in FTCs promotes dioxane degradation.

4C. Materials and Methods. FTCs consist of cylindrical reactors of 15 cm long and 4 cm diameter that are filled with ferrihydrite-coated sand prepared freshly. The reactors include inlet and outlet chambers designed to supply and collect the medium as a front across the entire section of the reactors that are separated from the porous medium by screens and $0.45\ \mu\text{m}$ pore-size membranes (Figure 1). The FTCs include a flow cell positioned in line with the outlet stream that can accommodate voltammetric microelectrodes to measure the composition of the outlet stream as a function of time with a high resolution [Beazley, M. J.; Webb, S. M.; Martinez, R. J.; Sobecky, P. A.; Taillefert, M. ,Carey, E. A.; Taillefert, M.]. Finally, outlet streams are sampled after the flow cell with a fraction collector for analysis of reactants and products (Figure 1). Using voltammetric Au/Hg microelectrodes with a DLK-70 Analytical Instrument Systems, Inc. (AIS) potentiostat and multiplexer that is operated autonomously, the concentration of dissolved oxygen ($\text{O}_{2(\text{aq})}$), and Fe^2 are monitored at each electrode simultaneously in the output flow with 10 minutes frequencies. In addition, these sensors are able to qualitatively detect the presence of soluble organic- Fe(III) complexes [Taillefert, M.; Bono, A. B.; Luther III, G. W.] which are produced during dissimilatory iron reduction [Jones, M.; Fennessey, C. M.; DiChristina, T. J.; Taillefert, M. ,Taillefert, M.; Beckler, J. S.; Carey, E. A.; Burns, J. L.; Fennessey, C. M.; DiChristina, T.]. Although these species cannot be quantified as the Fe(III) -complexing organic ligands [Taillefert, M.; Bono, A. B.; Luther III, G. W.] are unknown, they represent suitable proxies for microbial iron reduction [Beckler, J. S.; Kiriazis, N.; Stewart, F. J.; Rabouille, C.; Taillefert, M.]. The detection of organic- Fe(III) complexes in the output fluids therefore provides an indication that iron-reducing conditions dominate. Additional analyses conducted in the outlet fluids include: (i) inorganic anions and organic acids by ion chromatography [Dionex.]; (ii) H_2O_2 by horse radish peroxidase oxidation with ampliflu red as fluorescent indicator after stabilization in 100 mM diethylenetriaminepentaacetic acid (DTPA) to inhibit iron redox reactions [Dias, D. M. C.; Copeland, J. M.; Milliken, C. L.; Shi, X.; Ferry, J. L.; Shaw, T. J. ,Zhang, T.; Hansel, C. M.; Voelker, B. M.; Lamborg, C. H.]; and (iii) dioxane by HPLC [Scalia, S. ,Park, Y.-M.; Pyo, H.; Park, S.-J.; Park, S.-K.]. Solid phase analysis of the porous medium was obtained as a function of distance in the FTC at the end of the experiments using sequential chemical extraction techniques

[Kostka, J. E.; Luther III, G. W. ,Poulton, S. W.; Canfield, D. E.] to determine whether secondary minerals precipitated during the incubations.

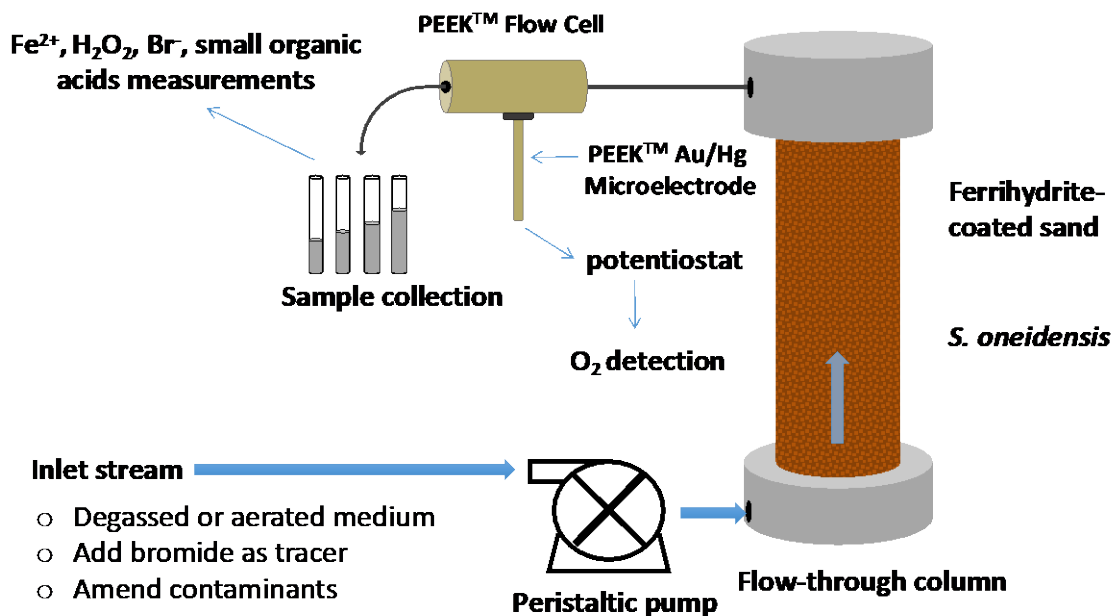


Figure 1. Flow-through column design and experiments. Cylindrical FTCs include inlet and outlet chambers designed to supply and collect medium as a front across the entire section of the reactor that are separated from the porous medium by screens and 0.45 mm pore size membranes. The geochemistry of the outlet stream is monitored over time with a voltammetric microelectrode in a flow cell positioned in line as well as via chemical analyses after collection with a fraction collector.

FTC experiments consisted of incubating *Shewanella oneidensis* strain MR-1 pre-immobilized homogeneously on iron oxide-coated sand across the entire reactors, running minimal media amended with reactants and Br as chemical tracer through the reactors while simultaneously measuring the redox chemical composition of the outlet streams. Five type of experiments were conducted in at least duplicates: (i) FTCs fed with aerobic media were compared to FTCs fed with anaerobic media in otherwise identical conditions to determine whether alternating aerobic and anaerobic phases are required in flow-through systems; (ii) Inlet streams amended with different nutrient concentrations to investigate the role of nutrient on the microbial response; (iii) FTCs amended with different cell concentrations to investigate the effect of the initial cell concentrations on the process efficiency; (iv) Inlet streams amended with different electron donor concentrations to optimize reactor conditions; and (v) FTCs operated in different flow conditions to determine the effect of the residence time of the inlet fluids on the microbially-driven Fenton reaction. In each of these treatments, duplicate FTCs were compared to abiotic control columns containing the solid medium but no cells. In all these experiments, bromide was injected periodically to calculate transport parameters and evaluate any effect due to the potential precipitation of reduced minerals on the porosity of these columns. The concentrations of dissolved O₂, Fe(II), H₂O₂, Br⁻, and electron donor respiration products were monitored over time at the output of the columns to follow the microbial reactions and the production of ROS. In addition, 1,4-dioxane and by-products of 1,4-dioxane degradation were determined at the output of each reactor to examine the degradation of the contaminant. Finally, net rates of reaction were determined by fitting the evolution of the

concentration of each species in the outlet fluids as a function of time with a transient one-dimensional reactive transport equation using a non-linear least square method [Beazley, M. J.; Webb, S. M.; Martinez, R. J.; Sobecky, P. A.; Taillefert, M. ,Carey, E. A.; Taillefert, M.].

To investigate the role of orthophosphate in the microbially-driven Fenton process, batch reactor incubations with 10^8 cells mL^{-1} *S. oneidensis* were conducted in duplicates in sealed serum bottles along with cell-unamended abiotic controls. A minimum medium with 20 mM lactate as electron donor and 10 mM ferrihydrite as electron acceptor was used in a M1 medium amended with 20 mM PIPES to maintain the pH around 7. Three different orthophosphate concentrations (50 μM , 1 mM, 9 mM) were used in the M1 medium to investigate the effect of phosphate on the microbially-driven Fenton reaction. For each incubation, the cell culture was allowed to reduce Fe(III) to a certain level under sealed conditions, and then was sparged with compressed air for 24 or 3 hours. The sealed and aerated periods were repeated several times thereafter referred to as redox cycles. Incubations were carried in two parallel yet identical cultures, and error bars indicate range of error between incubations.

At specific time-points about 2 mL samples of the uniformly suspended mixtures were removed and split in three aliquots, including 0.2 mL that was dissolved in 5 N HCl for total Fe(II) measurements [Taillefert, M., Beckler, J.S., Carey, E.A., Burns, J.L., Fennessey, C.M. and Di], 0.8 mL for immediate aqueous and mild-acid extractable (sorbed) Fe(II) measurements [Xiao, W., Jones, A.M., Collins, R.N., Bligh, M.W. and Waite, D.T.], and 1 ml immediately filtered with 0.22 μm MCE membrane filters (MF-Millipore™) for peroxide measurements using the same procedure as that of the FTC experiments. The samples collected for aqueous and mild-acid extractable (sorbed) Fe(II) were then micro-centrifuged (10,000 rpm, 60 s), and the aqueous Fe(II) content of the supernatant measured using the Ferrozine method [Stookey, L.L.]. The supernatant was then removed, 0.8 mL of 5 mM HCl added to the remaining solid sample, equilibrated for 30 s under agitation, and then micro-centrifuged as before to quantify mild acid-extractable (sorbed) Fe(II) in the supernatant by the Ferrozine method [Xiao, W., Jones, A.M., Collins, R.N., Bligh, M.W. and Waite, D.T.]. Structural Fe(II) was calculated by difference of total Fe(II) and the sum of aqueous and sorbed Fe(II). Concentration of hydroxyl radical was measured by the scavenger sodium benzoate (10 mM). The product of the reaction between benzoate and, p-HBA (p-hydroxybenzoic acid), was measured to estimate cumulative concentration with a conversion factor of 5.87 [Zhou, X.L. and Mopper, K.]. Concentrations of small organic acids concentrations (lactate, acetate and formate) were analyzed by ion chromatograph (IC) (Dionex DX-300 Series with CDM II conductivity detector) with a Dionex IonPac ICE-AS1 ion exclusion chromatography column and AMMS ICE II suppressor at a flow rate of 1.0 mL/min using 0.8 mM heptafluorobutyric acid as eluent and 5 mM tetrabutylammonium hydroxide as regenerant. Concentration of dissolved inorganic carbon (DIC) were analyzed by flow injection analysis (FIA) with conductivity detection [Hall, P.O.J. and Aller, R.C.] using a computer-operated Analytical Instruments Systems, Inc. (AIS, Inc) LCC100 integrator. At the end of the incubations, the reactors were sacrificed to collect the iron oxides through filtration, and the iron oxides were washed three times with medium and stored in air-tight container with oxygen scavenger until analyses. The crystalline phase of iron oxides after 2 cycles and 4 cycles was characterized by powder X-ray diffraction using a Panalytical Empyrean diffractometer with monochromatic Cu-K α radiation (λ = 0.15406 nm).

4D. Results and Discussion. *Flow-Through Column Experiments.* To sustain the microbially-driven Fenton reaction in the field for bioremediation efforts, it is imperative to determine whether redox conditions have to be oscillated over time. Dissolved oxygen measured at the output of the FTCs operated under different redox regime (oxic via aerated input solutions, anaerobic via degassed input solutions) demonstrated that the columns are deprived of dissolved oxygen after 8 days, even when fully aerated solutions are supplied to the solid medium (not shown). Dissolved Fe(II) concentrations as a function of time in the output fluids revealed that continuously aerated flow-through columns reduce ferrihydrite more efficiently than otherwise identical columns exposed to N₂-degassed media (Figure 2a), suggesting either that cell growth is more efficient in the aerated flow-through columns and/or that solid phase transformations sequester Fe(II) in the degassed columns. Although the microbial production of H₂O₂ was, as expected, higher in the

aerated columns (Figure 2b) the net production of acetate was similar in both conditions (Figure 2c).

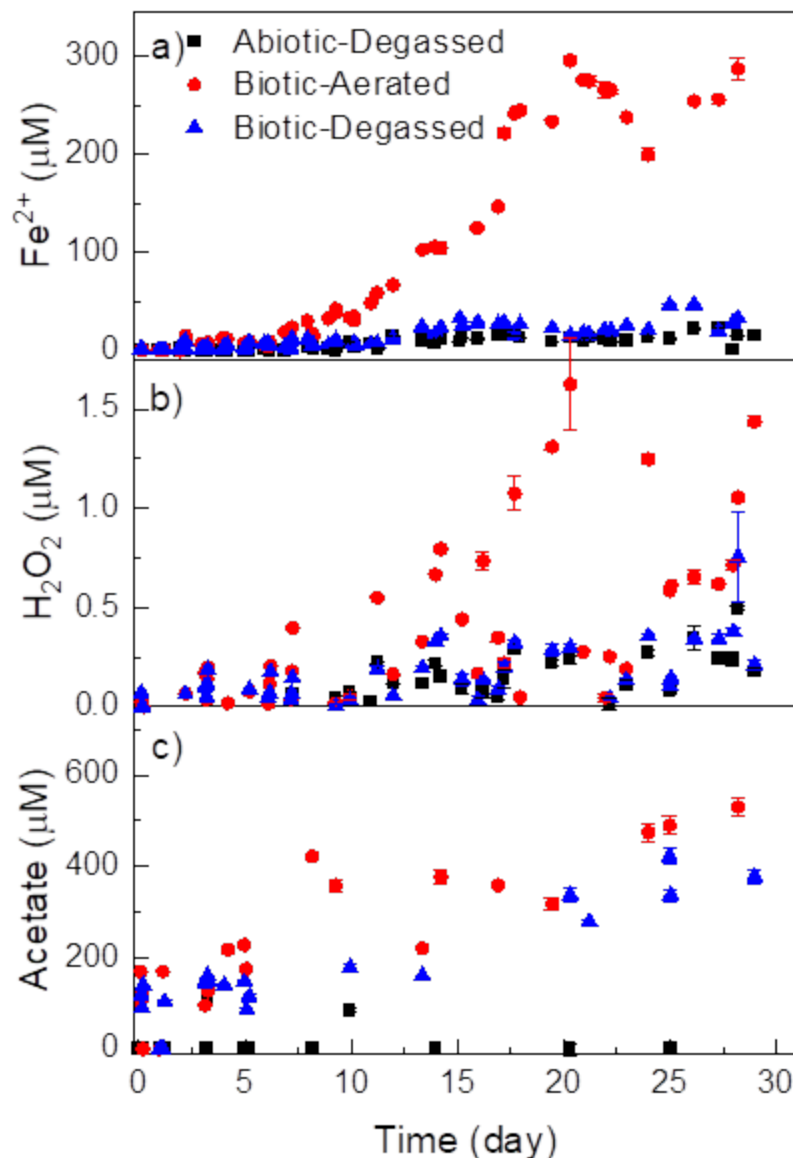


Figure 2. Variation in concentration of: a) dissolved Fe(II); b) H₂O₂; and c) Acetate as a function of time in the outlet fluids of FTCs either continuously amended with fully oxygenated (red) or deaerated (blue) medium in otherwise identical conditions relative to the abiotic control FTC. Cell concentrations: 10⁸ cells ml⁻¹; Medium: M1 amended with 5 mM Lactate as electron donor and 50 μM ΣPO₄³⁻ as nutrient at pH 7.0.

As acetate is used as electron donor by *S. oneidensis* in aerobic conditions only [Tang, Y.J.; Hwang, J.S.; Wemmer, D.E.; Keasling, J.D.; Pinchuk, G.E.; Geydebrekht, O.V.; Hill, E.A.; Reed, J.L.; Konopka, A.E.; Beliaev, A.], cell growth was likely more significant in the continuously

aerated flow-through columns. The different tracer injections revealed that the average porosity of the aerobic flow-through column changed over time (not shown), likely due to the precipitation of minerals, whereas breakthrough curves indicated that the precipitation of minerals was not significant in the anaerobic FTCs relative to the abiotic control column (not shown). Depending on the geochemical conditions, siderite or magnetite are well known by-product minerals from the microbial reduction of Fe(III) oxides by *S. oneidensis* [Zachara, J. M.; Kukkadapu, R. K.; Fredrickson, J. K.; Gorby, Y. A.; Smith, S. C. Bi-Hansel, C. M.; Benner, S. G.; Fendorf, S.]. The decrease in porosity of the solid medium and the higher production of Fe(II) at the output of the fully aerated flow-through columns therefore indicate that the reduction of the Fe(III) oxides was more efficient than in the anaerobic column. The surprisingly lower iron-reducing activity of the fully anaerobic column may be due to the generally lower growth- and ATP-yield of *S. oneidensis* under iron-reducing conditions compared to aerobic conditions [Roden, E.E.; Jin, Q.S. ,Jin, Q.S.] and the additional energy generated by the aerobic respiration of acetate. Overall, these findings indicate that the microbially-driven Fenton reaction can be sustained for at least a month long in FTCs and that alternative oxic and anoxic conditions are not necessary to promote the microbially-driven Fenton reaction, likely because a permanent redox gradient is established across the sediment column that promotes interaction of Fe(II) with H₂O₂. The intense reduction of Fe(III) oxides is also evident in the solid phase after the experiments, as the secondary mineralization of ferrihydrite to goethite [Zachara, J. M.; Kukkadapu, R. K.; Fredrickson, J. K.; Gorby, Y. A.; Smith, S. C. Bi,Hansel, C. M.; Benner, S. G.; Fendorf, S.] was promoted by the release of Fe(II) and the composition of the minimal medium (Figure 3). In fact, the secondary mineralization of ferrihydrite in the aerobic FTCs was almost as significant as that of the anaerobic FTCs.

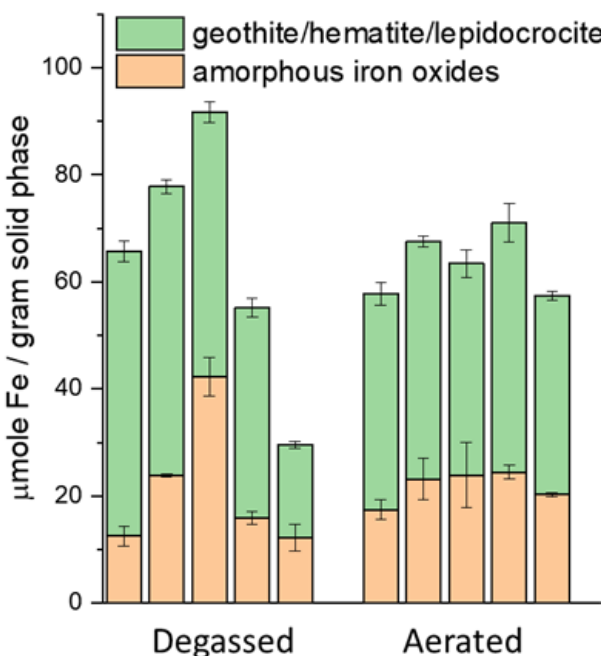


Figure 3. Speciation of Fe(III) oxides as a function of distance in the FTC reactors at the end of the experiment relative to the initial ferrihydrite material in: A) the degassed and B) fully aerated FTCs. Cell concentrations: 10⁸ cells ml⁻¹; Medium: M1 amended with 5 mM Lactate as electron donor and 50 mM SPO₄³⁻ as nutrient at pH 7.0; Flow Rate: 20 ml hr⁻¹.

The experimental conditions were then varied to optimize the microbially-driven Fenton process. First, the concentration of inorganic phosphate in the input medium was decreased from 9 mM in the original medium of batch incubations [Taillefert, M.; Beckler, J. S.; Carey, E. A.; Burns, J. L.; Fennessey, C. M.; DiChristina, T., Jones, M.; Fennessey, C.M.; DiChristina, T.J.; Taillefert, M.] to 20 μ M in FTC experiments to avoid potential interference of phosphate sorption onto ferrihydrite which could limit its dissolution [Stumm, W. Reactivity at the mineral-water interface: dissolution and inhibition.]. Net rates of Fe(II), H₂O₂, and acetate production at the output of the FTCs indicated that the concentration of inorganic phosphate in the input medium had to be raised to at least 50 μ M to facilitate growth of *S. oneidensis* (Figure 3). Net rates of Fe(II), H₂O₂, and acetate production were similar in subsequent column experiments employing 50 μ M inorganic phosphate in the medium, confirming the good reproducibility of our experimental setup. Second, a set of parallel FTC experiments was conducted to examine the effect of electron donor concentration on net rates of Fe(II) and H₂O₂ production. Net rates of Fe(II) and H₂O₂ production decreased significantly when lactate concentration in the inlet medium was decreased from 5 to 1 mM, but net rates of acetate production remained similar (Figure 4b).

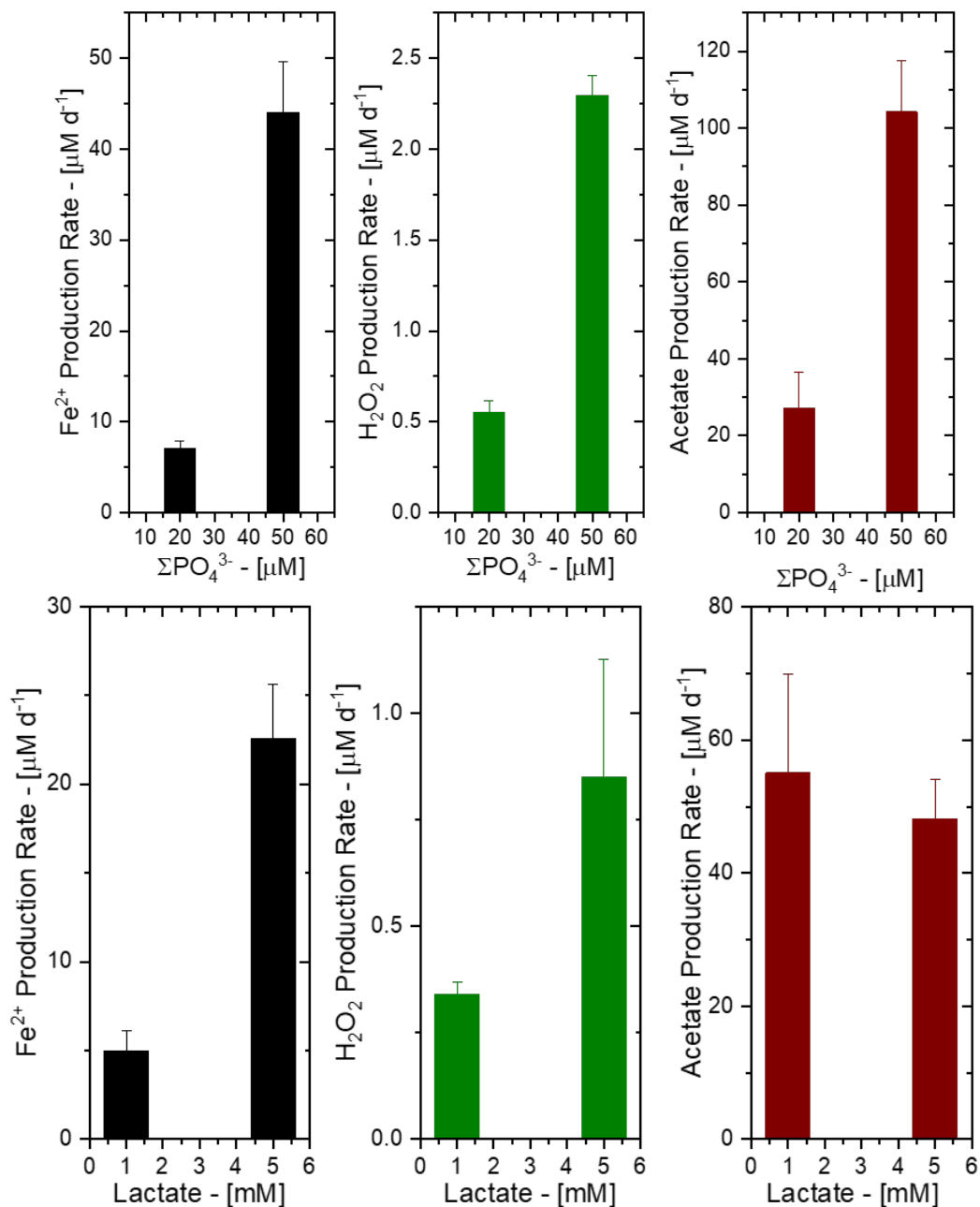


Figure 4. Effect of: a) phosphate and b) lactate concentrations in the medium on the net rate of: Fe(II), H₂O₂, and acetate production at the output of the FTCs. These experiments demonstrate that a minimum concentration of 50 μM orthophosphate is required to maintain growth of *S. oneidensis* in the FTCs. Cell concentrations: 10⁸ cells ml⁻¹; Medium: M1 amended with: a) 5 mM Lactate as electron donor and b) 50 μM ΣPO₄³⁻ at pH 7.0.

These findings suggest that a constant supply of 5 mM was enough to sustain microbial growth and that acetate consumption was actually enhanced in aerobic conditions at high lactate concentration, likely as a result of the significant increase in cell concentrations. Finally, the flow rate of the input solution into the FTCs was changed to examine its impact on microbially-driven Fenton reaction. Surprisingly, the net rate of both dissolved Fe(II) and H₂O₂ production increased

proportionally to the flow rate (Figure 5). Although net rate of H_2O_2 production was anticipated to increase as a function of the flow rate, due to the higher injection rate of dissolved oxygen in the reactors, it was expected that the net rate of Fe(II) production would decrease as a result of the simultaneous increase in Fe(II) oxidation rate and decrease in Fe(III) reduction rate in the reactors. These findings are surprising but the increase production of Fe(II) at higher flow rate may be due to the increase in biomass production as a result of the enhanced supply of dissolved oxygen which could also promote Fe(III) reduction near the output of the FTCs, where dissolved oxygen is depleted. Alternately, these findings could be explained by an increased rate of Fe(II) carbonate precipitation in the reactors as the flow rate decreases as a result of the overall higher metabolic activity of *S. oneidensis* at low flow rate. Indeed, a higher residence time of the pore waters may result in complete depletion of dissolved oxygen and higher rates of anaerobic Fe(III) respiration which both enhance carbonate and Fe(II) production, possibly above the saturation state of the solution with respect to siderite [Zachara, J. M.; Kukkadapu, R. K.; Fredrickson, J. K.; Gorby, Y. A.; Smith, S. C. Bi].

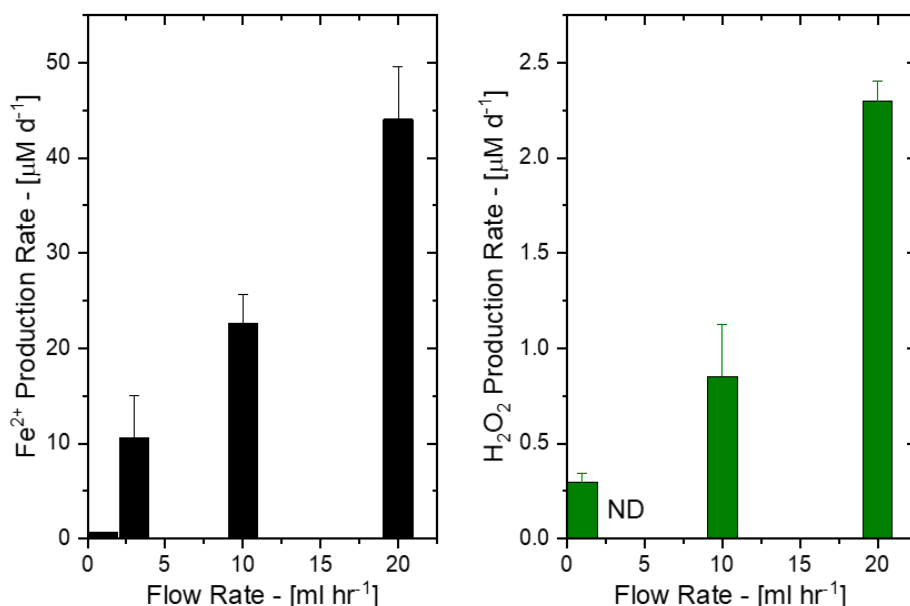


Figure 5. Effect of the flow rate on the net rate of: a) Fe(II) and b) H_2O_2 production during FTC experiments conducted with ferrihydrite-coated sand amended with *S. oneidensis* for 30 days in otherwise identical conditions. Cell concentrations: 10^8 cell ml^{-1} ; Medium M1 amended with 5 mM lactate as electron donor and $50 \mu\text{M } \Sigma\text{PO}_4^{3-}$ as nutrient at pH 7.0. ND means not determined.

The same FTCs loaded with ferrihydrite-coated sand and *S. oneidensis* were exposed to the contaminant 1,4-dioxane for about six weeks under fully aerated conditions to determine whether the generation of ROS in these conditions was able to promote the degradation of 1,4-dioxane. Two columns were continuously injected either with aerated or deaerated medium while 1,4-dioxane was added and removed twice from the input solution to simultaneously determine the effect of 1,4-dioxane on ROS production (Figure 6). Breakthrough curve of 1,4-dioxane revealed that a small fraction of 1,4-dioxane injected into the FTCs was removed from the input solutions,

likely via reaction with ROS species as the fraction removed was much higher in the fully aerated flow-through column compared to the degassed and abiotic control columns. Overall, these findings provide strong evidence for the microbial-driven Fenton reaction in flow-through systems, although the reaction may not be as efficient as originally achieved in batch reactors [Sekar, R.; DiChristina, T. J. ,Sekar, R.; Tallefert, M.; DiChristina, T. J.]. These findings suggest either that growth rates in the FTCs have to be optimized by altering nutrient (i.e., orthophosphate) concentrations or that Fe(III) oxyhydroxides are not as efficient as soluble Fe(III) substrates in promoting the microbially-driven Fenton reaction, possibly as a result of secondary mineralization processes that may deplete reactive Fe(III) from the system. These potential issues were investigated in batch reactor incubations.

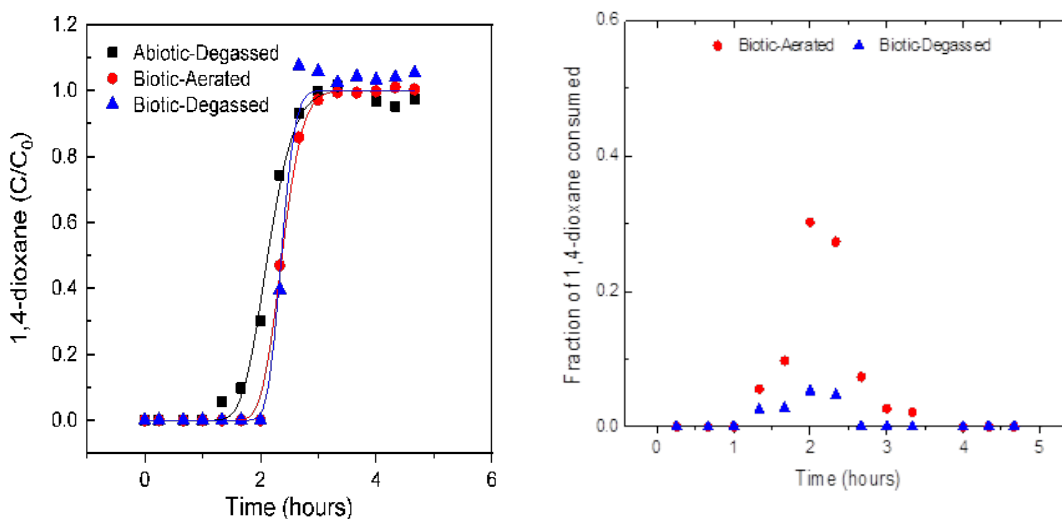


Figure 6. a) Breakthrough curve of 1,4-dioxane in biotic aerated and biotic degassed FTCs compared to the abiotic control. b) Resulting consumption of 1,4-dioxane relative to the abiotic control. The breakthrough curve of 1,4-dioxane is the most delayed for the biotic aerated column, followed by the biotic-degassed column relative to the abiotic control. These results indicate that 1,4-dioxane is partly degraded in the columns. However, the degradation efficiency is not optimum.

Role of Orthophosphate in the Microbially-Driven Fenton Reaction. The microbially-driven Fenton reaction and subsequent degradation of 1,4-dioxane was examined in batch experiments with iron oxyhydroxide as Fe(III) substrate and different orthophosphate concentrations to: (i) determine the role of inorganic phosphate in the microbially-driven Fenton process as phosphate is needed in the medium and generally low in subsurface environments; (ii) examine the importance of secondary mineralization in the microbially-driven Fenton process; and (iii) optimize the experimental conditions to maximize production of ROS and production of Fe(II).

Redox oscillations in the batch reactors were achieved by alternating between sealed and air-sparging periods during the course of the experiments (Figure 7). During the first sealed period (shown by the gray area), bacteria almost immediately consumed dissolved oxygen from the reactors and started to use ferrihydrite as terminal electron acceptor. Total Fe(II) production was similar during the first sealed period regardless of phosphate concentrations (Figure 7a), indicating

that inhibition of iron reduction by phosphate adsorption [Stumm, W. Reactivity at the mineral-water interface: dissolution and inhibition.] was not significant. In turn, the production rate of dissolved Fe(II) decreased proportionally to the orthophosphate concentrations (Figure 7b), and most Fe(II) was present in the form of mild acid-extractable (sorbed) (Figure 7c) or structural (Figure 7d) Fe(II). The lack of dissolved Fe(II) resulted from the formation of ferrous phosphate precipitates observed by x-ray diffraction (XRD) (not shown) and common in anaerobic incubations of *Shewanella oneidensis* MR1 [Borch, T.; Fendorf, S. Phosphate Interactions with Iron (Hydr)oxides: Mineralization Pa, Zachara, J. M.; Kukkadapu, R. K.; Fredrickson, J. K.; Gorby, Y. A.; Smith, S. C. Bi]. During the air-sparging phase, aqueous Fe(II) in all treatments was rapidly removed from solution (Figure 7b), likely by oxidation. Simultaneously, mild acid-extractable (sorbed) Fe(II) in the 9 mM orthophosphate incubations was oxidized at a slower rate compared to mild acid-extractable (sorbed) Fe(II) in the other two phosphate treatments (Figure 7c), further suggesting that ferrous phosphate precipitates, instead of sorbed ferrous ion, dominated the mild acid-extractable Fe(II) fraction in the 9 mM treatment. At the end of the aerated phase, structural Fe(II) was fully oxidized in the 50 μ M and 1 mM orthophosphate treatments (Figure 7d), whereas structural Fe(II) in the 9 mM orthophosphate treatment remained at a similar levels according to the resistance of ferrous phosphate precipitates to oxidation under oxic conditions [Nriagu, J. O. Stability of vivianite and ion-pair formation in the system Fe].

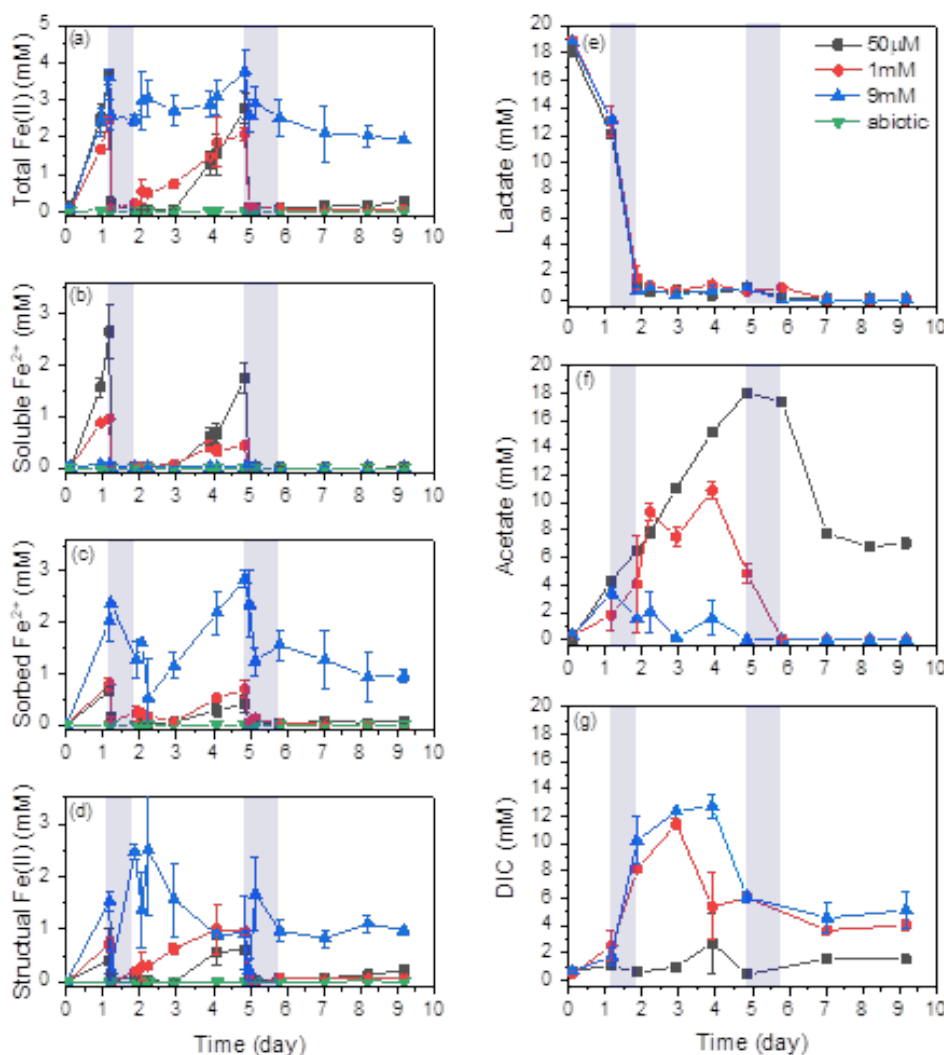


Figure 7. Iron speciation: (a) Total Fe(II), (b) soluble Fe(II), (c) sorbed Fe(II), and (d) structural Fe(II); and carbon transformation: (e) lactate, (f) acetate, and (g) dissolved inorganic carbon (DIC) by *S. oneidensis* as a function of time during redox cycles in M1 medium amended with lactate, ferrihydrite, and different orthophosphate concentrations (black squares, 50 μ M; red circles, 1 mM; blue triangles, 9 mM) relative to an abiotic control (green triangles). White areas correspond to the sealed periods, while blue shaded areas correspond to aerated periods. Concentrations and their error bars represent the average and standard deviations between duplicate incubations.

During the second sealed period, net total Fe(II) production decreased in all orthophosphate treatments, and iron reduction was even negligible during the third sealed period (Figure 7a), likely due to the lack of electron donor at that point of the incubations. Considering the similar acetate production during the different sealed periods (Figure 7f), the slower Fe(II) production rates were likely due to the lag phase required to switch metabolic process from aerobic respiration to anaerobic respiration of Fe(III). Interestingly, limited Fe(II) production was observed in the 9 mM orthophosphate treatment during the second seal period (Figure 7a). Newly produced Fe(II) mostly consisted of mild-acid extractable (sorbed) Fe(II) while structural Fe(II) remained mostly at the same level throughout the different cycles. Limited carbon sources could explain the limited Fe(II) production in the highest orthophosphate amendment.

Lactate was removed almost completely after two days (Figure 7e), while neither acetate (Figure 7f), formate (below detection limit), nor DIC (Figure 7g) were produced stoichiometrically. These findings indicate that *Shewanella* accumulated lactate before metabolizing it. Following this period, acetate was produced at rates inversely proportional to the orthophosphate concentration in the medium (Figure 7f), while DIC production rates increased proportionally to orthophosphate concentrations (Figure 7g). The TCA cycle represents the main pathway for oxygen consumption and biomass production in *Shewanella*, and acetate is completely mineralized during this process. In the absence or limited presence of oxygen, however, the TCA cycle is inhibited or truncated, and the intermediate acetyl-CoA is converted to acetate, the primary end-product of carbon catabolism during anaerobic growth of *S. oneidensis* [Tang, Y.J.; Hwang, J.S.; Wemmer, D.E.; Keasling, J.D. ,Rom]. In such conditions, acetate tends to accumulate given the strong preference of *S. oneidensis* for lactate but may be used when other carbon sources are depleted [Tang, Y.J.; Hwang, J.S.; Wemmer, D.E.; Keasling, J.D.]. The decrease in net acetate production and increase in DIC production as orthophosphate increased in the incubations therefore indicates that phosphate stimulated respiration in *S. oneidensis* MR-1 and that the lack of lactate forced the cells to respire on acetate, even in anaerobic conditions. The constant production of acetate under both the first aerated and second sealed periods (Figure 7f) in the 50 μ M and 1 mM phosphate incubations confirm the lactate preference of *S. oneidensis* MR-1 and suggests similar aerobic and anaerobic respiration rates. The removal of acetate after day 4 in the 1 mM and 9 mM phosphate incubations and after day 6 in the 50 μ M phosphate incubations confirm respiration of acetate as a result of the lack of lactate in the incubations. The simultaneous removal of DIC during that time period suggests precipitation of ferrous iron carbonates was significant during the incubations. Overall, higher orthophosphate concentrations in the medium increased respiration rates and likely promoted cell growth. The rapid consumption of lactate explains why iron reduction was not sustained during the third sealed period (Figure 7a), and subsequent experiments demonstrated that addition of lactate after the third redox oscillation was able to sustain the microbially-driven Fenton process (not shown).

Redox oscillations were also accompanied by a color change of the iron oxide suspensions (Figure 8). At the end of the first sealed period, the suspensions with 50 μ M and 1 mM orthophosphate were black (Figure 8a), while the suspension with 9 mM orthophosphate displayed a lighter orange color (Figure 8g) compared to the bright orange ferrihydrite in the abiotic controls (Figure 8c,f,i). After air-sparging, however, the 50 μ M and 1 mM orthophosphate suspensions became orange (Figure 8b,e) while the 9 mM orthophosphate suspension changed to a greenish brown color (Figure 8h). The color change of the iron oxides might be used as an indicator for the redox states of the reactors in large scale applications.

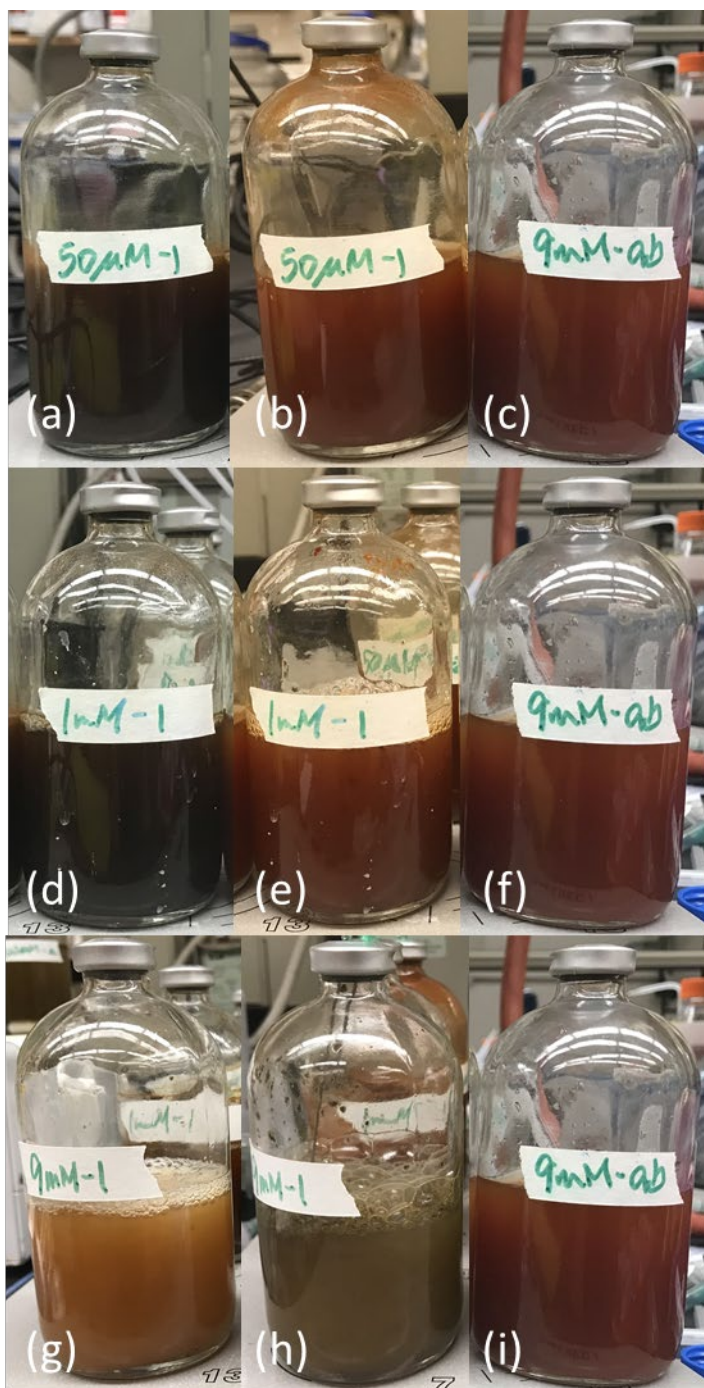


Figure 8. Color of the suspensions at the end of the first redox cycle during the microbially-driven Fenton reaction conducted with *S. oneidensis* in M1 medium amended with lactate, ferrihydrite, and: (a, b) 50 μ M; (d, e) 1 mM; and (g, h) 9 mM orthophosphate relative to otherwise identical incubations amended with: (c) 50 μ M; (f) 1 mM; and (i) 9mM orthophosphate without lactate to investigate the effect of microbial processes on the secondary mineralization of Fe(III) oxides.

Orthophosphate plays an important role in secondary mineral formation as phosphate adsorption stabilizes iron oxides [Hansel, C. M.; Benner, S. G.; Fendorf, S.]. Despite turning black at the end of the sealed periods, XRD analysis of the suspensions demonstrated that the solid phase

remained poorly crystallized after the first redox cycle in the 50 μM and 1 mM orthophosphate treatments (not shown). After two complete sealed-aerated cycles, the iron oxides in the 50 μM and 1 mM orthophosphate treatments remained relatively poorly crystallized (Figure 9a,b), while vivianite ($(\text{Fe}_3(\text{PO}_4)_2 \cdot n\text{H}_2\text{O})$) was detected in the 9 mM orthophosphate treatment (Figure 9c), likely because the vivianite solubility product was exceeded as a result of the respiration of Fe(III) oxides (Figure 7). After 4 complete redox cycles, iron oxides in the 1 mM orthophosphate medium remained poorly-crystallized (Figure 9b), whereas the 50 μM orthophosphate medium showed formation of goethite as secondary mineral (Figure 9a) and the 9 mM orthophosphate medium vivianite mainly (Figure 9c). The formation of vivianite is consistent with the observed color change of the suspension during the sealed-aerated cycles. The color of vivianite from white to colorless was previously observed under reducing conditions, while partially oxidized vivianite after air exposure changed to a blue color [Alborn, A.; Tomson, M.B.]. The color difference of the suspension between sealed and aerated periods suggest fresh vivianite was partially-oxidized by dissolved oxygen and the bacteria reduced the partially-oxidized vivianite during the sealed period. Vivianite oxidation proceeds slowly at room temperature and is stabilized by Fe(III) concentrations as high as 50% total Fe. Above 50% Fe(III), however, vivianite alters to metavivianite which is distinguished from vivianite by its X-ray diffraction spectrum [Rothe et al. 2016; Rouzies et al. 1993; Rothe et al. 2014]. In this study, blue oxidized vivianite remained stable at each redox cycle in the 9 mM orthophosphate treatment and less than 20% of total Fe(II) was oxidized during the aerated period. These conditions may therefore slow down the redox oscillation of Fe(III)/Fe(II) and affect the sustainability of the microbially-driven Fenton reaction compared to the low orthophosphate amendments which result in formation of goethite as secondary mineral. Adding lactate into the cell suspension with 9 mM orthophosphate to resume redox cycles produced more Fe(II) (Figure 7) and promoted ferrous phosphate precipitation with eventual consequences on the microbially-driven Fenton process. Overall, 1 mM orthophosphate may represent the best condition of the three orthophosphate concentrations used for the sustainability of the microbially-driven Fenton reaction for several redox cycles, as orthophosphate stabilizes the surface of iron oxides and keeps it poorly crystallized [Cornell, R.M.; Giovanoli, R.].

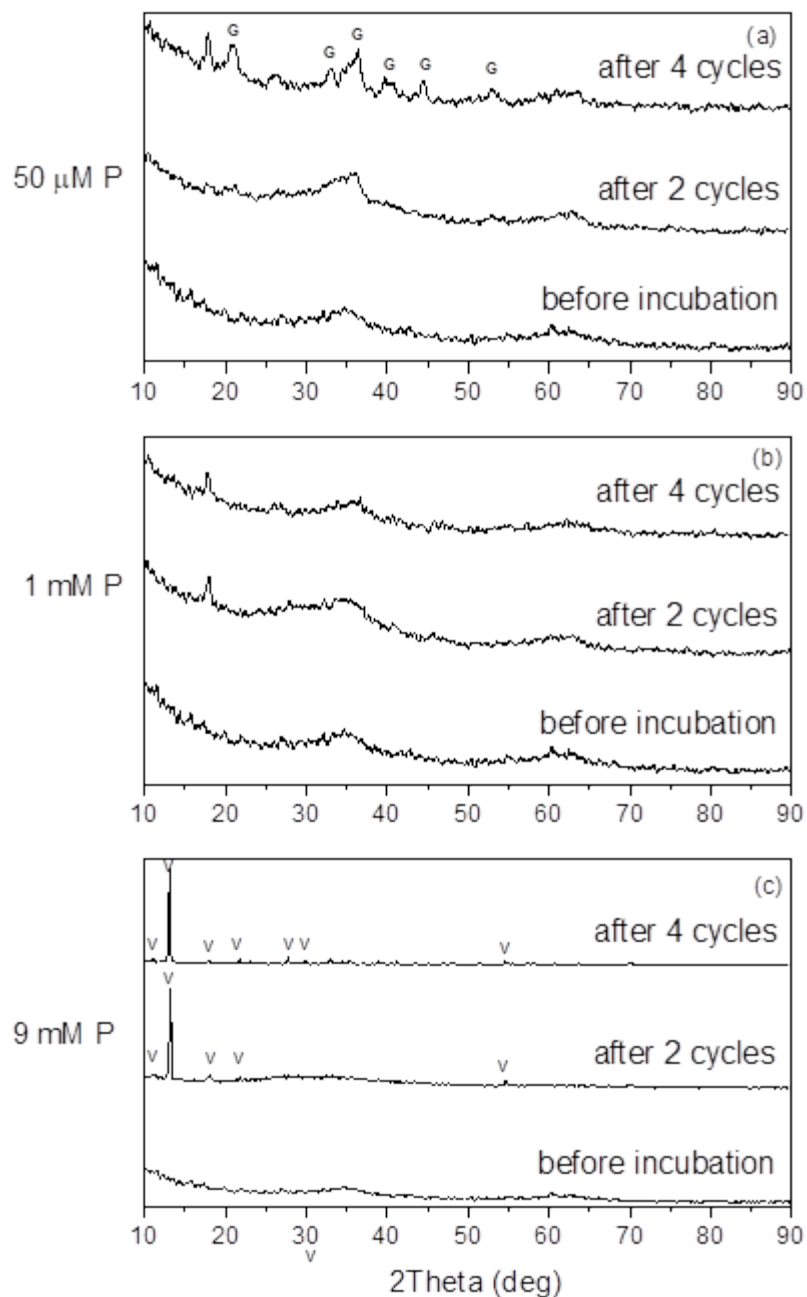


Figure 9. Secondary mineralization during the microbially-driven Fenton reaction. X-ray diffraction patterns of the solid phase from *S. oneidensis* incubations in M1 medium amended with lactate, ferrihydrite, and three different orthophosphate concentrations: (a) 50 μM ; (b) 1 mM; and (c) 9 mM after exposure to different redox cycles compared to before the incubation. G and V represent goethite and vivianite identified using solid phase standards.

Hydrogen peroxide (H_2O_2) was produced during the first two sealed periods and was removed during the air-sparging periods (Figure 10). In addition, H_2O_2 concentrations produced (Figure 10) and rates of H_2O_2 production obtained during various redox cycle experiments (Figure 11b) were always the highest in the 1 mM orthophosphate treatment, suggesting that this treatment has the highest capacity to generate the Fenton reaction for the degradation of organic contaminants. No

net H_2O_2 production was observed during the third sealed period, however, likely due the low metabolic activity as a result of the depletion of electron donor. In general, H_2O_2 is generated extracellularly as intermediate during the biological reduction of dissolved oxygen [Messner, K.R.; Imlay, J.A.] or is produced by chemical oxidation of Fe(II) by dissolved oxygen [Rush, J.D.; Bielski, B.H.J.].

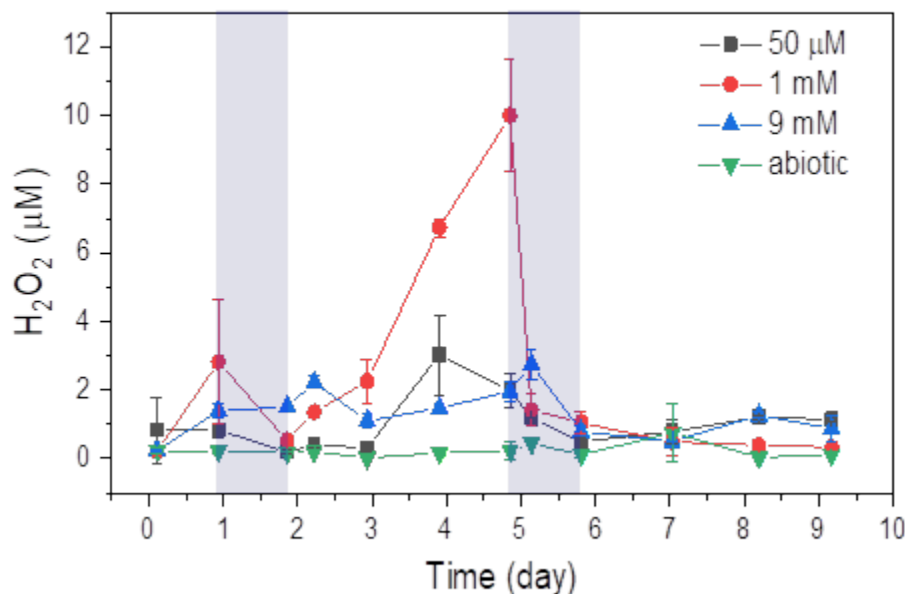


Figure 10. Hydrogen peroxides (H_2O_2) concentrations produced by *S. oneidensis* as a function of time during redox cycles in M1 medium amended with lactate, ferrihydrite, and different orthophosphate concentrations (black squares, 50 μM ; red circles, 1 mM; blue triangles, 9 mM) relative to an abiotic control (green triangles). White areas correspond to the sealed periods, while blue shaded areas correspond to aerated periods. Concentrations and their error bars represent the average and standard deviations between duplicate incubations.

Separate abiotic experiments (not shown) demonstrated that abiotic Fe(II) oxidation is not able to generate enough H_2O_2 under similar conditions as the incubations conducted in this study, suggesting that microbial production represents the main H_2O_2 generation pathway during the microbially-driven Fenton reaction [Sekar, R.; DiChristina, T. J.]. The low H_2O_2 concentrations detected during the air-sparging periods relative to the sealed periods may be explained by the expression of catalases or peroxidases to cope with oxidative stress as a result of the sudden redox changes experienced in these experiments [Mishra, S.; Imlay, J.A. ,Li, N.; Luo, Q.X.; Jiang, Y.M.; Wu, G.F.; Gao, H.C.]. In turn, the overall increase in net H_2O_2 production rates during the sealed phase of the second redox cycle that was reproduced in subsequent incubations (not shown) cannot be attributed to the incomplete microbial respiration of dissolved oxygen as dissolved oxygen was likely depleted during this phase dominated by iron reduction. This behavior is supported by the rebound in iron reduction and net H_2O_2 production rates by lactate addition before a fourth redox cycle conducted in replicate incubations after repletion of lactate (Figure 11). These findings suggest that an alternative mechanism is responsible for the production of H_2O_2 in anaerobic conditions.

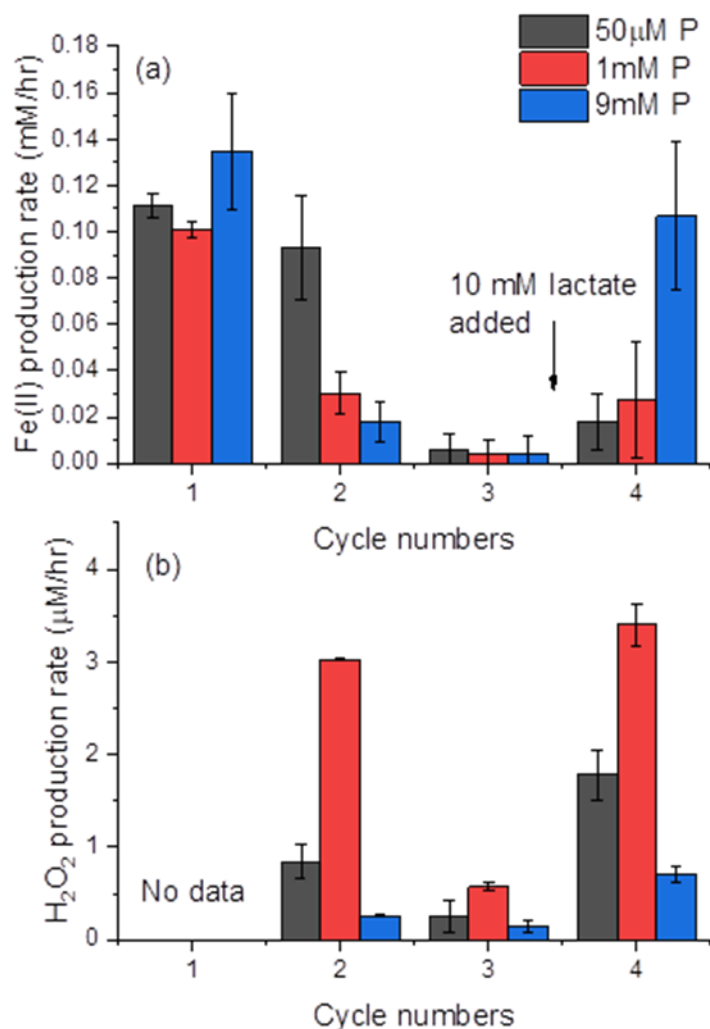
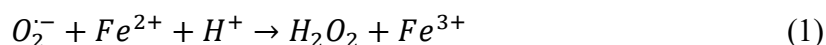


Figure 11. Net production rates of: (a) Total Fe(II) and (b) H₂O₂ by *S. oneidensis* during up to four redox cycles in M1 medium amended with lactate, ferrihydrite, and different orthophosphate concentrations (black bars, 50 μM; red bars, 1 mM; blue bars, 9 mM). Error bars represent the standard deviations of the average rates of duplicate incubations.

Mechanism of ROS Production in the Microbially-Driven Fenton Reaction. H₂O₂ is one of the metabolic by-products of cellular oxygen respiration and is mostly produced intracellularly through accidental auto-oxidation of non-respiratory flavoproteins and the turnover of committed oxidases or directly generated at the cell surface by amino acid oxidases during metabolism of exogenous organic nitrogen sources [Imlay, J.A. -Gu, C.; Wang, J.; Guo, M.F.; Sui, M.; Lu, H.; Liu, G.F.]. Once produced, intracellular H₂O₂ may diffuse across the outer membrane into the medium [Imlay, J.A.]. Hydrogen peroxide, however, was not detected (detection limit 0.2 μM, data not shown) in the *S. oneidensis* cell suspension (10⁸ cells/mL) with the modified medium under aerobic conditions in this study (Fe species removed), suggesting that bacterial production of H₂O₂ was not significant. Alternately, H₂O₂ is readily produced abiotically by reduction of superoxide by reductants such as Fe(II) (Eq. 1) [Rose, A.L.; Waite, D.T. ,Burns, J.M.; Craig, P.S.; Shaw, T.J.; Ferry, J.L.].

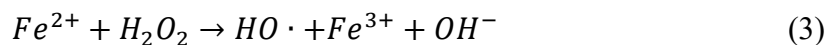


In turn, production of extracellular superoxide is widespread under aerobic conditions in heterotrophic bacteria, including *S. oneidensis* [Han, R.X.; Lv, J.T.; Huang, Z.Q.; Zhang, S.H.; Zhang, S.Z.], and likely catalyzed by cell surface NAD(P)H oxidoreductases [Diaz, J.M.; Hansel, C.M.; Voelker, B.M.; Mendes, C.M.; Andeer, P.F.; Zhang, T.]. In addition, superoxide may also be produced during the abiotic oxidation of Fe(II) by dissolved O₂ (Eq. 2) [Rose, A.L.; Waite, D.T. ,Burns, J.M.; Craig, P.S.; Shaw, T.J.; Ferry, J.L.].

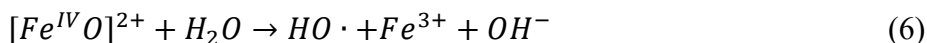
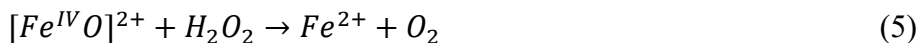
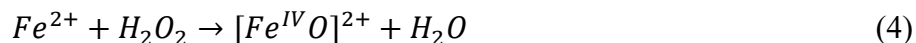


Thus, the lack of H₂O₂ production in aerobic *S. oneidensis* cultures and the formation of high H₂O₂ concentrations at the end of the seal periods suggest that the oxidation of Fe(II) by superoxide (Eq. 1) may represent a significant source of H₂O₂ during the microbially-driven Fenton reactions. A similar metastable Fe(II), O₂, superoxide, and H₂O₂ mixture was reported in sediment pore waters where positive correlation between Fe(II) and H₂O₂ suggested abiotic ROS production [Dias, D. M. C.; Copeland, J. M.; Milliken, C. L.; Shi, X.; Ferry, J. L.; Shaw, T. J.].

Despite extensive studies in the past few decades, the detailed mechanism of the Fenton reaction is not fully understood. In particular, the identity of the active intermediate has long been debated [Bataineh, H.; Pestovsky, O.; Bakac, A.]. There are two generally accepted mechanisms, including the Harber-Weiss mechanism in which hydroxyl radicals are produced via the one-electron reduction of H₂O₂ by ferrous iron (Eq. 3) [Barb, W.G.; Baxendale, J.H.; George, P.; Hargrave, K.R.].



The hydroxyl radical is a strong oxidant with a high reduction potential (E⁰ = +2.59 V [Remucal, C.K.; Sedlak, D.L.]) that is able to react at near-diffusion controlled rate with various reduced species to terminate ROS regeneration [Neyens, E.; Baeyens, J.A.] and potentially cause cell damage to *S. oneidensis* [Ghosal, D.; Omelchenko, M.V.; Gaidamakova, E.K.; Matrosova, K.S.; Wackett, L.P.; Fr]. The sustainability of the microbially-driven Fenton reaction, therefore, suggests the involvement of a non-radical pathway such as the two-electron transfer reduction of H₂O₂ by Fe(II) accompanied by the formation of the high-valent ferryl(IV)-oxo intermediate (Eq. 4) which eventually is reduced back to Fe(II) by H₂O₂ (Eq. 5) [Bataineh, H.; Pestovsky, O.; Bakac, A. ,Bray, W.C.; Gorin, M.H. ,Kremer, M.L.]. The net reaction in these pathways involves the conversion of H₂O₂ into H₂O and O₂ by cycling iron between Fe(II) and Fe(IV) without the production of hydroxyl radical. The ferryl-oxo intermediate may alternately hydrolyze and produce hydroxyl radical (Eq. 6) which makes that pathway equivalent to the Harber-Weiss mechanism (Eq. 3).



In general, the ferryl iron ion is a weaker and more selective oxidant (E⁰ = +1.80 V [Dunford, H.B. ,He, J.; Yang, X.F.; Men, B.; Wang, D.S.]) that has a longer lifetime (~0.35 s) in solution compared to the hydroxyl radical (half-life of a few nanoseconds [Bacic, G.; Mojovic, M.]). The Fenton-produced ferryl(IV)-oxo intermediate was identified in a non-complexing buffer at near-neutral pH [Bataineh, H.; Pestovsky, O.; Bakac, A. ,Pham, A.L.T.; Lee, C.; Doyle, F.M.];

Sedlak, D.L. -Keenan, C.R.; Sedlak, D.L.], whereas the hydroxyl radical was the main intermediate in a phosphate buffer [Bataineh, H.; Pestovsky, O.; Bakac, A.]. Indeed, abiotic control incubations with Fe(II) and H₂O₂ in a 9 mM orthophosphate medium showed much higher hydroxyl radical production efficiency than identical experiments in a 50 M and 1 mM orthophosphate medium (Figure 12). These data imply the formation of the non-radical intermediate may be responsible for the higher H₂O₂ accumulation rates in the low orthophosphate amendments (Figure 11b). In addition, the lower H₂O₂ production rates in the 50 μ M than 1 mM orthophosphate incubations may be explained by differences in cell growth, as evidenced by the production of acetate and DIC (Figure 7). Overall, these findings suggest that the phosphate ligand environment drives the Fenton reaction into different pathways, resulting either in the production of hydroxyl radicals or catalytic decomposition to O₂ that may further catalyze the formation of H₂O₂ under anaerobic conditions via reduction of O₂ to superoxide intermediate.

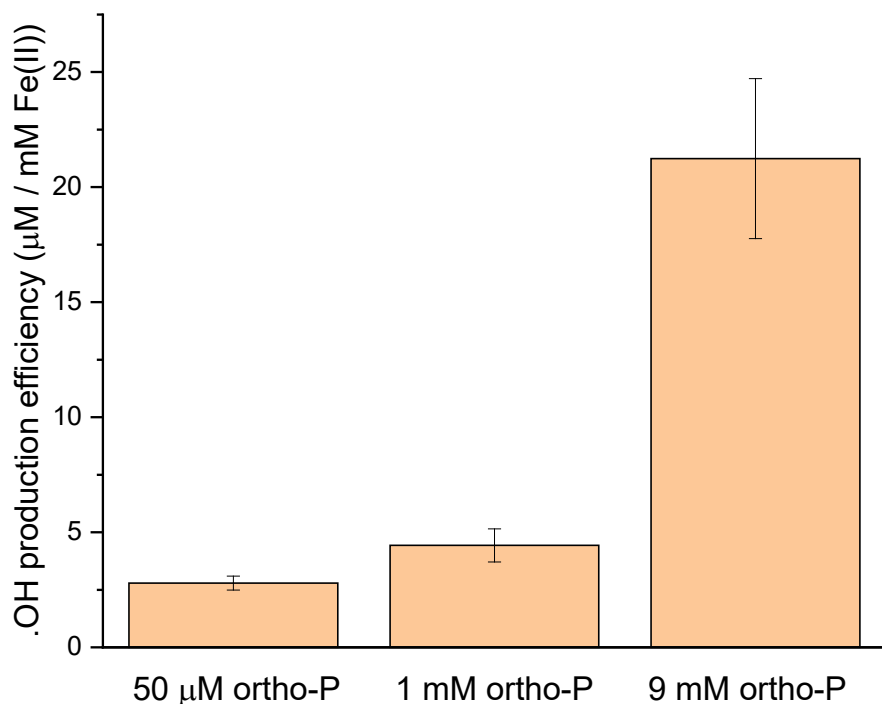


Figure 12. Hydroxyl radical production efficiency ($\mu\text{M} \cdot \text{OH} / \text{mM Fe(II)}$ oxidized) of the abiotic Fenton reaction in the presence of different orthophosphate concentrations. Error bars represent the standard deviations between triplicate experiments.

The iron-catalyzed ROS cryptic cycle that is proposed to take place in the microbially-driven Fenton process is provided in Figure 13. In the beginning of the sealed period, high O₂ and low Fe(II) concentrations may lead to the direct microbial production of superoxide. As O₂ becomes depleted, cells switch to Fe(III) reduction which produces Fe(II) that may successively react with superoxide to generate H₂O₂ (Eq. 1), thus resulting in an overall positive correlation between soluble Fe(II) and H₂O₂ ($R^2 = 0.460$ and $R^2 = 0.642$ in the 50 μM and 1 mM orthophosphate

media, data not shown). It is likely that progressively increasing Fe(II) concentrations may favor the abiotic reduction of H_2O_2 to O_2 with formation of the ferryl intermediate in the low orthophosphate media (Eq. 4 and Eq. 5) and formation of hydroxyl radical in the 9 mM orthophosphate medium (Eq. 3), generating an overall negative correlation between soluble Fe(II) and H_2O_2 concentrations in the low orthophosphate media ($R^2 = 0.617$ and $R^2 = 0.623$ in the 50 μM and 1 mM, data not shown) but not in the 9 mM orthophosphate medium ($R^2 = 0.055$). The anaerobic generation of O_2 in the low orthophosphate media may then promote a catalytic process that involves either the microbially- or abiotically-produced (Eq. 2) superoxide that enhances abiotic production of H_2O_2 by Fe(II) oxidation (Eq. 1) in a cryptic ROS cycle. Net accumulation of H_2O_2 during the sealed period indicates that the rate-limiting step in this process must be H_2O_2 reduction (Eq. 3-5). When the electron donor is exhausted or the reactors are switched back to the aerated phase, Fe(III) reduction ceases and the cryptic ROS cycle eventually breaks down due to the lack of Fe(II) production.

Figure 13. Proposed iron-catalyzed ROS cryptic cycling taking place during the microbially-driven Fenton reaction. Abiotic processes are in black, whereas biological processes are in blue.

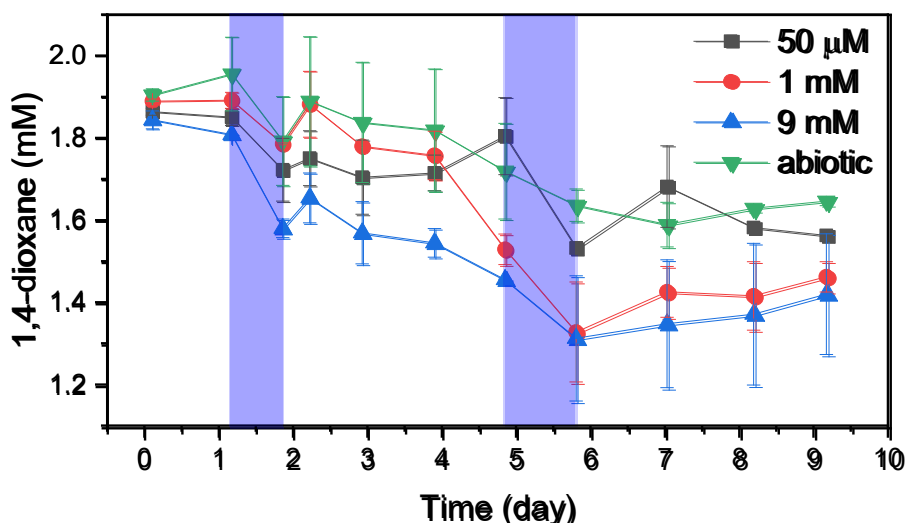


Figure 14. 1,4-dioxane degradation by *S. oneidensis* as a function of time during redox cycles in M1 medium amended with different orthophosphate concentrations (black squares, 50 μ M; red circles, 1 mM; blue triangles, 9 mM) relative to an abiotic control (green triangles). White areas correspond to the sealed periods, while blue shaded areas correspond to aerated periods. Concentrations and their error bars represent the average and standard deviations between duplicate incubations.

Besides the effect of phosphate on cell metabolism and the production of H_2O_2 by the cells, complexation of orthophosphate to Fe(II) in circumneutral conditions may facilitate the Fenton reaction and promote the generation of hydroxyl radicals [Bataineh, H.; Pestovsky, O.; Bakac, A.]. In fact, orthophosphate coordination to Fe(II) lowers the activation energy of the reaction with H_2O_2 and promotes the production of hydroxyl radical as opposed to the formation of the less reactive ferryl complex (Fe(IV)) without orthophosphate coordination. Compared to the 9 mM orthophosphate medium, the microbially-driven Fenton reaction with 1 mM orthophosphate degraded similar levels of 1,4-dioxane (22 $\pm$ 3%) by the end of the incubations, but the rate of 2,4-dioxane degradation was much lower during the first redox cycle and much higher during the second redox cycle, the period corresponding to the highest H_2O_2 production rates observed during the incubations (Figure 11b). Overall, the microbially-driven Fenton reaction with Fe(III) oxides in M1 medium containing 1 mM orthophosphate promotes bacterial growth, produces higher H_2O_2 concentrations in solution, and degrades more 1,4-dioxane with a more sustainable redox cycling given the lack of secondary minerals formed in these conditions. In turn, these degradation rates are not as efficient as those achieved with ferric citrate as terminal electron acceptor [Sekar, R.; DiChristina, T. J., Sekar, R.; Taillefert, M.; DiChristina, T. J.], suggesting that the stabilization of Fe(II) by organic ligands may help enhance the efficiency of the microbially-driven Fenton degradation of organic contaminants.

Role of Organic Ligands in the Microbially-Driven Fenton Reaction. Although the microbially-driven Fenton reaction can be sustained with ferrihydrite as iron substrate, 1,4-dioxane degradation rates were consistently much lower than with ferric citrate as iron source. To investigate the impact of the ligand citrate on Fe(III) reduction and 1,4-dioxane degradation, duplicate batch incubations of *S. oneidensis* with 10 mM Fe(III)-citrate or 2-line ferrihydrite were conducted in parallel and compared to an abiotic control in otherwise identical conditions.

Reactors were sealed until Fe(II) reached a certain level, then purged with air for 3 hours, and these processes were repeated four times. Although more Fe(II) was produced during the first 2 hours of the incubation with soluble citrate-Fe(III) than with ferrihydrite as terminal electron acceptor (Figure 15a), similar Fe(III) reduction rates were observed during the next three redox cycles. These findings suggest the formation of the same ferric oxyhydroxides phases in the Fe(III)-citrate incubations after the first reoxidation phase. Despite similar Fe(II) production rates, the degradation of 1,4-dioxane was significantly enhanced in the Fe(III)-citrate incubations compared to the ferrihydrite incubations (Figure 15b), with more than 36% 1,4-dioxane degraded by the microbially-driven Fenton reaction after four redox cycles. Organic ligands such as citrate affect the mechanism and kinetics of the Fenton reaction and enhances hydroxyl radical production [Illes, E.; Patra, S.G.; Marks, V.; Mizrahi, A.; Meyerstein, D.; Zeng, B.; Zhang, P.; Zheng, M.; Xiao, N.; Han, J.; Wang, C.; Wang, Z.; Zhao, Z.], which may explain the higher degradation rates of 1,4-dioxane observed in these incubations. The stabilization of Fe(II) in solution by the organic ligand may also enhance the generation of hydroxyl radicals, but this has yet to be investigated.

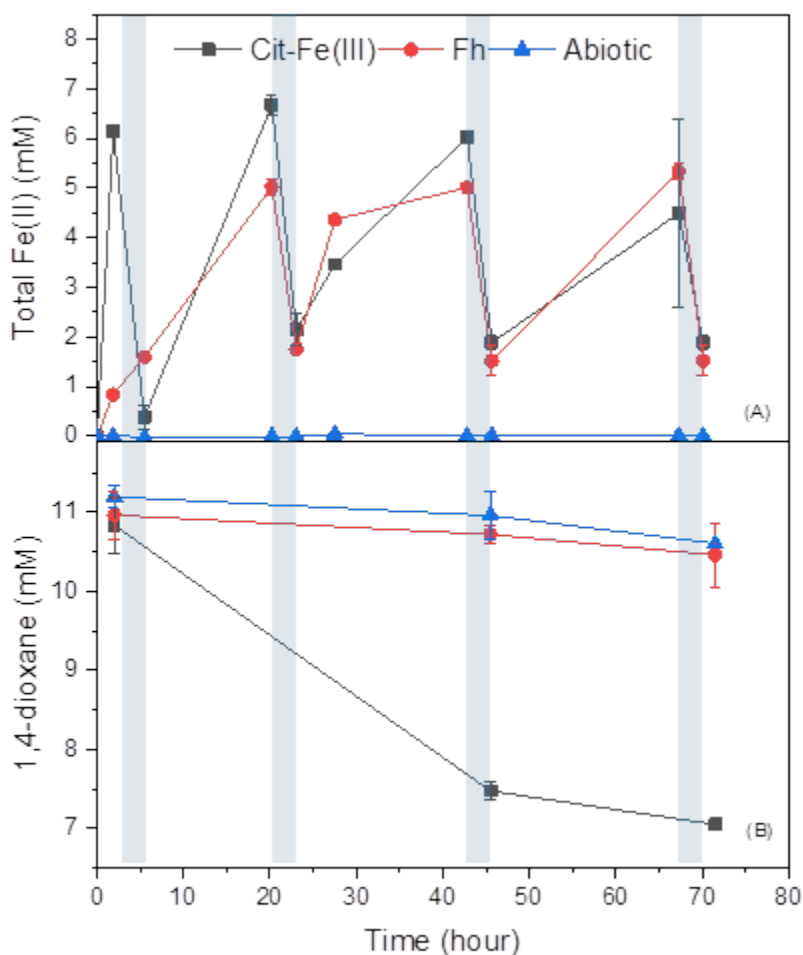


Figure 15. Total Fe(II) (A) and 1,4-dioxane (B) concentrations as a function of time during redox cycles in *S. oneidensis* incubations in M1 medium amended with different Fe(III) sources as terminal electron

acceptor (black squares, ferric citrate; red circles, 2-line ferrihydrite) compared to an abiotic control (blue triangles). Blue shaded areas correspond to three-hour long aerated periods, whereas white areas represent the sealed periods. Incubations were carried out in two parallel yet identical cultures, and error bars represent the standard deviation between the duplicate incubations.

4E. Conclusions and Implications for Future Research. In this study, flow through column and batch reactor incubations were used to investigate whether iron oxyhydroxides could be used as terminal electron acceptor by *Shewanella oneidensis* MR-1 to produce Fe(II) that could be eventually exploited in the microbially-driven Fenton reaction to degrade organic contaminants. FTC experiments demonstrated that oscillations between aerobic and anaerobic conditions were not necessary to generate the microbially-driven Fenton reaction, likely because a redox gradient was established across the reactors where both H_2O_2 and Fe(II) were generated. These experiments also demonstrated that the flow rate of the input solution, nutrient, and electron donor concentrations have to be optimized to maximize the efficiency of the microbially-driven Fenton reaction. The degradation of 1,4-dioxane was established in the FTCs and was more efficient in aerated columns than columns not exposed to dissolved oxygen, providing strong evidence for the microbial-driven Fenton reaction in flow-through systems, although the reaction may not be as efficient as originally achieved in batch reactors. These findings suggest either that growth rates in FTCs have to be optimized by altering nutrient (i.e., orthophosphate) concentrations or that Fe(III) oxyhydroxides are not as efficient as soluble Fe(III) substrates in promoting the microbially-driven Fenton reaction, possibly as a result of secondary mineralization processes that may deplete reactive Fe(III) from the system. These potential issues were investigated in batch reactor incubations using redox oscillations to be able to promote the microbially-driven Fenton reaction with *S. oneidensis*.

Batch reactor incubations using up to four complete redox cycles demonstrated that enough electron donor was required to sustain the microbially-driven Fenton reaction, that lactate was rapidly taken up by the cells before respiration, and that low electron donor conditions may favor oxidative degradation of acetate by *S. oneidensis*. More importantly, batch reactor incubations demonstrated that higher orthophosphate concentrations are preferred to enhance bacterial growth in the reactors and that most of this growth appears to occur during the aerated phase of the incubations. In addition, high orthophosphate concentrations are preferred to promote the microbially-driven Fenton reaction, as low orthophosphate concentrations promote production of ferryl-ion intermediates that may decrease the production of hydroxyl radicals. In turn, high orthophosphate concentrations promote precipitation of iron phosphate minerals as secondary mineral products that may limit the recycling of Fe(III) oxyhydroxides. Thus, orthophosphate concentrations that are sufficiently high to promote growth and generate hydroxyl radicals are required to be able to sustain the microbially-driven Fenton reaction but not enough to exceed the solubility product of vivianite and other iron phosphate minerals which may scavenge iron and decrease the sustainability of the reaction. Secondary mineralization to goethite was evident in the low orthophosphate incubations and did not appear to prevent the microbially-driven Fenton reaction.

Batch reactor experiments also provided new insights into the mechanism of the microbially-driven Fenton reaction. It appears that direct production of H_2O_2 by reduction of dissolved O_2 by *S. oneidensis* during the aerated phase is not significant. Instead, most of the H_2O_2 produced occurs during the sealed period by a cryptic ROS cycle that seems to involve the

microbial production of superoxide at the beginning of the sealed phase, when dissolved O₂ is still available. As O₂ is consumed, *S. oneidensis* switches to Fe(III) reduction which produces Fe(II) that may successively react with superoxide to generate H₂O₂. The abiotic reduction of H₂O₂ to O₂ with formation of the ferryl intermediate may be favored in the low orthophosphate media, whereas formation of hydroxyl radical is most significant in high orthophosphate conditions. The anaerobic generation of O₂ in the low orthophosphate media may then promote a catalytic process that involves either the microbially- or abiotically-produced superoxide that enhances abiotic production of H₂O₂ by Fe(II) oxidation in a cryptic ROS cycle. Net accumulation of H₂O₂ during the sealed period indicates that the rate-limiting step in this process must be H₂O₂ reduction. When the electron donor is exhausted or the reactors are switched back to the aerated phase, Fe(III) reduction ceases and the cryptic ROS cycle eventually breaks down due to the lack of Fe(II) production.

Finally, batch reactor experiments demonstrated that 1,4-dioxane degradation by the microbially-driven Fenton reaction may not be as efficient with ferrihydrite than ferric citrate as terminal electron acceptor, even though recycling of Fe(III) during the first oxidative cycle in the ferric citrate incubations resulted in the production of an iron oxyhydroxide phase with the same reactivity as in the ferrihydrite incubations. As organic ligands are known to affect the Fenton reaction, it is likely that stabilization of Fe(II) by ligands in solution as well as participation of the organic ligand in dark ROS production affects the generation of hydroxyl radicals that are involved in organic contaminant degradation. The pandemic prevented to test these hypotheses more thoroughly. Future studies will compare incubations with and without citrate and other naturally-occurring organic ligands (i.e. humics) to determine whether the presence of organic ligands may help improve the efficiency of the microbially-driven Fenton degradation of organic contaminants in flow-through column experiments. These findings will ultimately indicate whether the microbially-driven Fenton reaction occurs naturally in subsurface environments, potentially contributing to natural attenuation processes. In addition, the results from the flow-through column experiments conducted in this study demonstrate that the microbially-driven Fenton reaction is sustainable without high frequency redox oscillations, thus allowing us in future studies to transfer this technology to the pump-and-treat of contaminated subsurface waters.

4F. References

1. Sekar, R.; DiChristina, T. J. **2014**. Microbially driven Fenton reaction for degradation of the widespread environmental contaminant 1,4-dioxane. *Environmental Science and Technology*. 48, 12858-12867.
2. Sekar, R.; Taillefert, M.; DiChristina, T. J. **2016**. Simultaneous transformation of comingled trichloroethylene, tetrachloroethylene, and 1,4-Dioxane by a microbially driven Fenton reaction in batch liquid cultures. *Applied and Environmental Microbiology*. 82, 6335-6343.
3. Stumm, W. Reactivity at the mineral-water interface: dissolution and inhibition. *Coll. Surf. A*, **1997**, 120: 143-166.
4. Borch, T.; Fendorf, S. Phosphate Interactions with Iron (Hydr)oxides: Mineralization Pathways and Phosphorus Retention upon Bioreduction. *Dev. Earth Env. Sci.* **2008**, 7, 321-348.

5. Zachara, J. M.; Kukkadapu, R. K.; Fredrickson, J. K.; Gorby, Y. A.; Smith, S. C. Biomineralization of poorly crystalline Fe(III) oxides by dissimilatory metal reducing bacteria (DMRB). *Geomicrobiol. J.* **2002**, *19*, 179-207.
6. Hansel, C.M.; Benner, S.G.; Neiss, J.; Dohnalkova, A.; Kukkadapu, R.K.; Fendorf, S. **2003** Secondary mineralization pathways induced by dissimilatory iron reduction of ferrihydrite under advective flow. *Geochim. Cosmochim. Acta* *67*, 2977-2992.
7. Hansel, C. M.; Benner, S. G.; Fendorf, S. **2005** Competing Fe(II)-induced mineralization pathways of ferrihydrite. *Environ. Sci. Technol.* *39*, 7147-7153.
8. Beazley, M. J.; Webb, S. M.; Martinez, R. J.; Sobecky, P. A.; Taillefert, M. **2011**. The effect of pH and natural microbial phosphatase activity on the speciation of uranium in subsurface soils. *Geochim. Cosmochim. Acta.* *75*, 5648-5663.
9. Carey, E. A.; Taillefert, M. **2005**. The role of soluble Fe(III) in the cycling of iron and sulfur in coastal marine sediments. *Limnol. Oceanogr.* *50*, 1129-1141.
10. Taillefert, M.; Bono, A. B.; Luther III, G. W. **2000**. Reactivity of Freshly Formed Fe(III) in Synthetic Solutions and (Pore)Waters: Voltammetric Evidence of an Aging Process. *Environ. Sci. Technol.* *34*, 2169-2177.
11. Jones, M.; Fennessey, C. M.; DiChristina, T. J.; Taillefert, M. **2010**. *Shewanella oneidensis* MR-1 mutants selected for their inability to produce soluble organic-Fe(III) complexes are unable to respire Fe(III) as anaerobic electron acceptor. *Environ. Microbiol.* *12*, 938-950.
12. Taillefert, M.; Beckler, J. S.; Carey, E. A.; Burns, J. L.; Fennessey, C. M.; DiChristina, T. J. **2007**. *Shewanella putrefaciens* produces an Fe(III)-solubilizing ligand during anaerobic respiration on insoluble Fe(III) oxides *J. Inorg. Biochem.* *101*, 1760-1767.
13. Beckler, J. S.; Kiriazis, N.; Stewart, F. J.; Rabouille, C.; Taillefert, M. **2016**. Importance of microbial iron reduction in deep sediments of river-dominated continental margins. *Mar. Chem.* *178*, 22-34.
14. Dionex. **2011**. Determination of organic acids in wastewater using ion-exclusion chromatography and on-Line carbonate removal. 291, 1-6.
15. Dias, D. M. C.; Copeland, J. M.; Milliken, C. L.; Shi, X.; Ferry, J. L.; Shaw, T. J. **2016**. Production of reactive oxygen species in the rhizosphere of a *Spartina*-dominated salt marsh system. *Aquat. Geochem.* *22*, 573-591.
16. Zhang, T.; Hansel, C. M.; Voelker, B. M.; Lamborg, C. H. **2016**. Extensive dark biological production of reactive oxygen species in brackish and freshwater ponds. *Environ. Sci. Technol.* *50*, 2983-2993.
17. Scalia, S. **1990**. Reversed-phase high-performance liquid chromatographic method for the assay of 1, 4-dioxane in sulphated polyoxyethylene alcohol surfactants. *J. Pharm. Biomed. Anal.* *8*, 867-870.
18. Park, Y.-M.; Pyo, H.; Park, S.-J.; Park, S.-K. **2005**. Development of the analytical method for 1, 4-dioxane in water by liquid-liquid extraction. *Anal. Chim. Acta.* *548*, 109-115.

19. Kostka, J. E.; Luther III, G. W. **1994**. Partitioning and speciation of solid phase iron in saltmarsh sediments. *Geochim. Cosmochim. Acta*. 58, 1701-1710.
20. Poulton, S. W.; Canfield, D. E. **2005**. Development of a sequential extraction procedure for iron: implications for iron partitioning in continentally derived particles. *Chem. Geol.* 214, 209-221.
21. Taillefert, M., Beckler, J.S., Carey, E.A., Burns, J.L., Fennessey, C.M. and DiChristina, T.J. **2007** *Shewanella putrefaciens* produces an Fe(III)-solubilizing ligand during anaerobic respiration on insoluble Fe(III) oxides. *J. Inorg. Biochem.* 101, 1760-1767.
22. Xiao, W., Jones, A.M., Collins, R.N., Bligh, M.W. and Waite, D.T. **2017** Use of Fourier transform infrared spectroscopy to examine the Fe(II)-catalyzed transformation of ferrihydrite. *Talanta* 175, 30-37.
23. Stookey, L.L. **1970**. Ferrozine: a new spectrophotometric reagent for iron. *Anal. Chem.* 42, 779-781.
24. Zhou, X.L. and Mopper, K. **1990** Determination of photochemically produced hydroxyl radicals in seawater and freshwater. *Mar. Chem.* 30, 71-88.
25. Hall, P.O.J. and Aller, R.C. **1992** Rapid, small-volume, flow injection analysis for $\square\text{CO}_2$ and NH_4^+ in marine and freshwaters. *Limnol. Oceanogr.* 37, 1113-1119.
26. Tang, Y.J.; Hwang, J.S.; Wemmer, D.E.; Keasling, J.D. **2007** *Shewanella oneidensis* MR-1 fluxome under various oxygen conditions. *Appl. Environ. Microbiol.* 73, 718-729.
27. Pinchuk, G.E.; Geydebrekht, O.V.; Hill, E.A.; Reed, J.L.; Konopka, A.E.; Beliaev, A.S.; Frederickson, J.K. **2011** Pyruvate and lactate metabolism by *Shewanella oneidensis* MR-1 under fermentation, oxygen limitation, and fumarate respiration conditions. *Appl. Environ. Microbiol.* 77, 8234-8240.
28. Roden, E.E.; Jin, Q.S. **2011** Thermodynamics of microbial growth coupled to metabolism of glucose, ethanol, short-chain organic acids, and hydrogen. *Appl. Environ. Microbiol.* 77, 1907-1909.
29. Jin, Q.S. **2012** Energy conservation of anaerobic respiration. *Amer. J. Sci.* 312, 573-628.
30. Jones, M.; Fennessey, C.M.; DiChristina, T.J.; Taillefert, M. **2010** *Shewanella oneidensis* MR-1 mutants selected for their inability to produce soluble organic-Fe(III) complexes are unable to respire Fe(III) as anaerobic electron acceptor. *Environ. Microbiol.* 12, 938-950.
31. Nriagu, J. O. Stability of vivianite and ion-pair formation in the system $\text{Fe}_3(\text{PO}_4)_2\text{-H}_3\text{PO}_4\text{-H}_3\text{PO}_4\text{-H}_2\text{O}$. *Geochim. Cosmochim. Acta*. **1972**, 36, 459-470.
32. Romanek, C.S.; Zhang, C.L.L.; Li, Y.L.; Horita, J.; Vali, H.; Cole, D.R.; Phelps, T.J. **2003** Carbon and hydrogen isotope fractionations associated with dissimilatory iron-reducing bacteria. *Chem. Geol.* 195, 5-16.
33. Hunt, K.A.; Flynn, J.M.; Naranjo, B.; Shikhare, I.D.; Gralnick, J.A. **2010** Substrate-level phosphorylation is the primary source of energy conservation during anaerobic respiration of *Shewanella oneidensis* Strain MR-1. *J. Bacteriol.* 192, 3345-3351.

34. Alborno, A.; Tomson, M.B. **1994** The temperature dependence of the solubility product constant of vivianite. *Geochim. Cosmochim. Acta* 58, 5373-5378.
35. Rothe, M.; Kleeber, A.; Hupfer, M. **2016** The occurrence, identification and environmental relevance of vivianite in waterlogged soils and aquatic sediments. *Earth Sci. Rev.* 158, 51-64.
36. Rouzies, D.; Millet, J.M.M. **1993** Mossbauer study of synthetic oxidized vivianite at room temperature. *Hyperf. Interact.* 77, 19-28.
37. Rothe, M.; Friedrichs, T.; Eder, M.; Kleeber, A.; Hupfer, M. **2014** Evidence for vivianite formation and its contribution to long-term phosphorus retention in a recent lake sediment: a novel analytical approach. *Biogeosci.* 11, 5169-5180.
38. Cornell, R.M.; Giovanoli, R. **1985** Effect of solution conditions on the proportion and morphology of goethite formed from ferrihydrite. *Clays Clay Miner.* 33, 424-432.
39. Messner, K.R.; Imlay, J.A. **1999** The identification of primary sites of superoxide and hydrogen peroxide formation in the aerobic respiratory chain and sulfite reductase complex of *Escherichia coli*. *J. Biol. Chem.* 274, 10119-10128.
40. Rush, J.D.; Bielski, B.H.J. **1985** Pulse radiolytic studies of the reactions of H₂O₂/O₂⁻ with Fe(II)/Fe(III) ions. The reactivity of H₂O₂/O₂⁻ with ferric ions and its implications on the occurrence of the Haber-Weiss reaction. *J. Phys. Chem.* 89, 5062-5066.
41. Mishra, S.; Imlay, J.A. **2012** Why do bacteria use so many enzymes to scavenge hydrogen peroxide? *Arch. Biochem. Biophys.* 525, 145-160.
42. Li, N.; Luo, Q.X.; Jiang, Y.M.; Wu, G.F.; Gao, H.C. **2014** Managing oxidative stresses in *Shewanella oneidensis*: intertwined roles of the OxyR and OhrR regulons. *Environ. Microbiol.* 16, 1821-1834.
43. Imlay, J.A. **2013** The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. *Nat. Rev. Microbiol.* 11, 443-454.
44. Diaz, J.M.; Plummer, S. **2018** Production of extracellular reactive oxygen species by phytoplankton: past and future directions. *J. Plankton Res.* 40, 655-666.
45. Gu, C.; Wang, J.; Guo, M.F.; Sui, M.; Lu, H.; Liu, G.F. **2018** Extracellular degradation of tetrabromobisphenol A via biogenic reactive oxygen species by a marine *Pseudoalteromonas* sp. *Wat. Res.* 142, 354-362.
46. Rose, A.L.; Waite, D.T. **2002** Kinetic model for Fe(II) oxidation in seawater in the absence and presence of natural organic matter. *Environ. Sci. Technol.* 36, 433-444.
47. Burns, J.M.; Craig, P.S.; Shaw, T.J.; Ferry, J.L. **2010** Multivariate examination of Fe(II)/Fe(III) cycling and consequent hydroxyl radical generation. *Environ. Sci. Technol.* 44, 7226-7231.
48. Han, R.X.; Lv, J.T.; Huang, Z.Q.; Zhang, S.H.; Zhang, S.Z. **2020** Pathway for the production of hydroxyl radicals during the microbially mediated redox transformation of iron (oxyhydr)oxides. *Environ. Sci. Technol.* 54, 902-910.

49. Diaz, J.M.; Hansel, C.M.; Voelker, B.M.; Mendes, C.M.; Andeer, P.F.; Zhang, T. **2013** Widespread production of extracellular superoxide by heterotrophic bacteria. *Science* 340, 1223-1226.
50. Bataineh, H.; Pestovsky, O.; Bakac, A. **2012** pH-induced mechanistic changeover from hydroxyl radicals to iron(IV) in the Fenton reaction. *Chem. Sci.* 3, 1594-1599.
51. Barb, W.G.; Baxendale, J.H.; George, P.; Hargrave, K.R. **1951** Reactions of ferrous and ferric ions with hydrogen peroxide. Part I. - The ferrous ion reaction. *Trans. Farad. Soc.* 47, 462-500.
52. Remucal, C.K.; Sedlak, D.L. **2011** The role of iron coordination in the production of reactive oxidants from ferrous iron oxidation by oxygen and hydrogen peroxide, in: Tratnyek, P.G., Grundl, T.J., Haderlein, S.B. (Eds.), *Aquatic Redox Chemistry*. American Chemical Society, pp. 177-197.
53. Neyens, E.; Baeyens, J.A. **2003** A review of classic Fenton's peroxidation as an advanced oxidation technique. *J. Hazard. Mat.* 98, 33-50.
54. Ghosal, D.; Omelchenko, M.V.; Gaidamakova, E.K.; Matrosova, K.S.; Wackett, L.P.; Frederickson, J.K.; Daly, M.J. **2005** How radiation kills cells: Survival of *Deinococcus radiodurans* and *Shewanella oneidensis* under oxidative stress. *FEMS Microbiol. Rev.* 29, 361-375.
55. Bray, W.C.; Gorin, M.H. **1932** Ferryl ion, a compound of tetravalent iron. *J. Amer. Chem. Soc.* 54, 2124-2125.
56. Kremer, M.L. **1999** Mechanism of the Fenton reaction. Evidence for a new intermediate. *Phys. Chem. Chem. Phys.* 1, 3595-3605.
57. Dunford, H.B. **2002** Oxidation of iron (II)/(III) by hydrogen peroxide: from aquo to enzyme. *Coord. Chem. Rev.* 233, 311-318.
58. He, J.; Yang, X.F.; Men, B.; Wang, D.S. **2016** Interfacial mechanisms of heterogeneous Fenton reactions catalyzed by iron-based materials: A review. *J. Environ. Sci. China* 39, 97-109.
59. Bacic, G.; Mojovic, M. **2005** EPR spin trapping of oxygen radicals in plants - A methodological overview. *Ann. N. Y. Acad. Sci.* 1048, 230-243.
60. Pham, A.L.T.; Lee, C.; Doyle, F.M.; Sedlak, D.L. **2009** A silica-supported iron oxide catalyst capable of activating hydrogen peroxide at neutral pH values. *Environ. Sci. Technol.* 43, 8930-8935.
61. Katsoyiannis, I.A.; Ruettimann, T.; Hug, S.J. **2008** pH dependence of Fenton reagent generation and As(III) oxidation and removal by corrosion of zero valent iron in aerated water. *Environ. Sci. Technol.* 42, 7424-7430.
62. Keenan, C.R.; Sedlak, D.L. **2008** Factors affecting the yield of oxidants from the reaction of nanoparticulate zero-valent iron and oxygen. *Environ. Sci. Technol.* 42, 1262-1267.
63. Illes, E.; Patra, S.G.; Marks, V.; Mizrahi, A.; Meyerstein, D. **2020** The FeII(citrate) Fenton reaction under physiological conditions. *J. Inorg. Biochem.* 206, 111018: 1-7.

64. Zeng, B.; Zhang, P.; Zheng, M.; Xiao, N.; Han, J.; Wang, C.; Wang, Z.; Zhao, Z. **2019** Detection and identification of the oxidizing species generated from the physiologically important Fenton-like reaction of iron (II)-citrate with hydrogen peroxide. *Arch. Biochem. Biophys.* 668, 39-45.

5. Enhancement of the Microbially-Driven Fenton Reaction via Genetic Manipulation of *Shewanella* Genes Involved in Oxidative Stress Response.

5A. Objective. The major objective of this work is to enhance the rate of the Fenton reaction and thereby the rate of contaminant degradation by manipulating select genes directly implicated in ROS defense in the Fe(III)-reducing facultative anaerobe *Shewanella oneidensis*, including catalases ($2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2$) and peroxidases ($\text{ROOR}' + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{ROH} + \text{R}'\text{OH}$).

5B. Background. The microbially driven Fenton reaction produces hydroxyl radicals ($\text{OH}^\bullet$) that degrade a wide range of environmental contaminants including pentachlorophenol, 1,4-dioxane, trichloroethylene, perchloroethylene, pyrene, and anthracene (1-4). In contrast to conventional (purely abiotic) Fenton remediation, microbially driven Fenton operates at a circumneutral pH, does not require exogenous addition of reagents, and regenerates catalysts. MFR is a one-pot bioreactor containing minimal growth medium amended with ferric iron, various environmental pollutants, and *S. oneidensis* MR-1. The bioreactor is initially degassed to allow anaerobic ferric iron respiration by *S. oneidensis* MR-1, which reduce all available Fe(III) to Fe(II). After all iron is reduced, the reactor is sparged with compressed air and cells will switch to aerobic respiration, which produces hydrogen peroxide (H_2O_2) and superoxide as waste products. Ferrous iron (Fe(II)) and H_2O_2 react to produce the hydroxyl radical $\text{HO}^\bullet$, which initiates a chain of radical reactions, attacking and degrading the environmental pollutants.

The conditions inside the microbially-driven Fenton bioreactors that allow for oxidation of contaminants would seem inhospitable to microbes, but *S. oneidensis* MR-1 are able to complete several (4-5) cycles before contaminant degradation rate or population are affected (2, 3, 4). In addition to the hydroxyl radical produced by the Fenton reaction, other reactive oxygen species (ROS) formed by the cell include peroxides and superoxide, which are byproducts of incomplete metabolism of molecular oxygen during oxidative phosphorylation in cytochrome C oxidase during aerobic respiration. ROS are generally harmful to cells due to radical propagation reactions, which can damage biomolecules like DNA, proteins, and lipid oxidation, causing cell death (5).

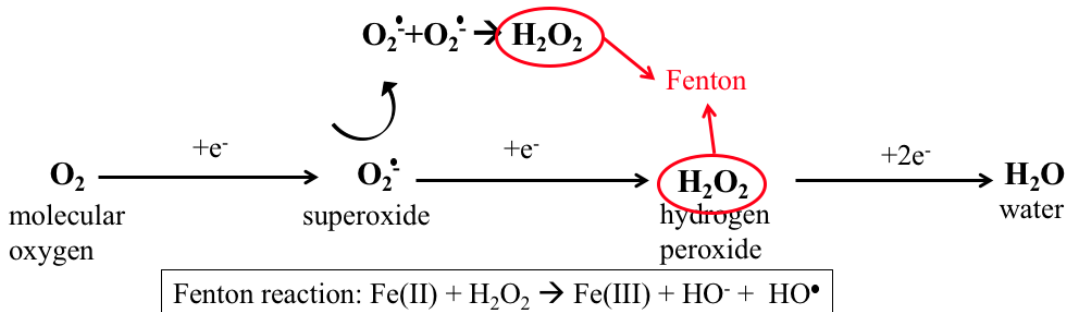


Fig 1. Metabolism of molecular oxygen during oxidative phosphorylation.

Molecular oxygen is small and uncharged, and diffuses across membranes quickly enough to where cells cannot meaningfully lower their intracellular O_2 concentration substantially below the extracellular O_2 concentration (6). In contrast to O_2 , exogenous H_2O_2 crosses the outer membrane via porin proteins into the periplasm, where it passively diffuses through the inner membrane to the cytoplasm (7,8). Exogenous H_2O_2 arises from various sources: the chemical oxidation of reduced metals at sediment-water interfaces, including iron; the photochemical formation of oxidants by flavins; and excretion by lactic acid bacteria and plants attempting to

suppress growth of competitors and dominate resources (9). Major endogenous sources of ROS are generated when molecular oxygen oxidizes menaquinones or solvent-exposed FAD or FMN on flavoenzymes (autooxidation), especially fumarate reductase, generating destructive superoxide ($O_2^{\cdot-}$) and H_2O_2 (10-14). While H_2O_2 is semi membrane-permeable, intracellular concentrations are tightly controlled and H_2O_2 influx into cells is limited by the rate of membrane diffusion (12).

Consequently, many aerobic and facultative microbes, and even strict anaerobe species which live at sediment-water interfaces or other zones of high redox stress, evolved strategies to protect themselves from intracellular ROS. Upon sensing organic peroxides or H_2O_2 , *S. oneidensis* upregulates expression of operons *ohrR* and *oxyR*, respectively (15). *ohrR* controls expression of the major organic peroxide scavenger Ohr, and periplasmic glutathione peroxidase *pgpD* (SO_1563) (15). OxyR, a transcription factor, directly senses H_2O_2 and acts as the primary regulator of oxidative stress in *S. oneidensis* MR-1. OxyR regulates over 20 genes involved in ROS detoxification by upregulating or derepressing expression of genes whose products directly scavenge free radicals, such as catalase and superoxide dismutase, and genes that indirectly affect the intracellular ROS content, like Dps, which sequesters intracellular free Fe(II) to avoid intracellular Fenton reaction and thus formation of intracellular hydroxyl radical (16). When intracellular H_2O_2 is low, alkylhydroperoxidase enzyme is the major scavenger in *E. coli* (9). The enzymes catalase and peroxidase directly protect the cell from H_2O_2 and organic peroxides when intracellular concentrations are high by catalyzing their conversion into water and molecular oxygen, while superoxide dismutase disproportionates superoxide radicals into water and H_2O_2 , which is often subsequently removed by catalase (Fig. 2). *S. oneidensis* MR-1 encodes several ROS scavenging genes: 1 dedicated catalase, 2 catalase-peroxidases, 6 dedicated peroxidases, and 1 superoxide dismutase.

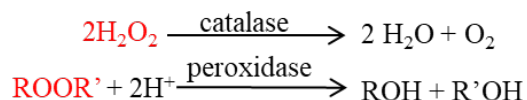


Figure 2. Catalase- and peroxidase-catalyzed reactions.

Although *S. oneidensis* naturally occupy sediment-water interfaces and other redox-active zones, they are highly sensitive to oxidative stress and radiation, which generates intracellular ROS (15, 17-19). However, *S. oneidensis* are well-known for their ability to respire on an extensive range of terminal electron acceptors, which has applications for bioremediation of heavy metal/radionuclide contaminated soil and groundwater. While the vast anaerobic respiratory networks responsible for inorganic and metal respiration have been elaborated in detail, comparatively little has been studied about how *S. oneidensis* respond to oxidative stress during aerobic respiration, which is especially relevant during the aerobic cycles of the microbially driven Fenton reaction. If more is understood about how *S. oneidensis* protect themselves from oxidative stress, it may be possible to enhance contaminant degradation rates. A detailed investigation of the *S. oneidensis* MR-1 cellular response to oxidative stress is required to develop a more complete understanding of the role existing cellular defense mechanisms against ROS play in both 1) protecting the cells from oxidative stress during microbially-driven Fenton reaction, and 2) facilitating the microbially-driven Fenton reaction.

5C. Materials and Methods. In-frame gene deletion. Ten in-frame single and double gene deletion mutants related to oxidative stress were constructed in *Shewanella oneidensis* MR-1. One

dedicated catalase (SO_1070, $\Delta katB$), two bifunctional catalase-peroxidases (SO_0725, $\Delta katG2$; SO_4405, $\Delta katG1$), and periplasmic glutathione peroxidase (SO_3349, $\Delta pgpD$) were deleted from *S. oneidensis* MR-1. Double deletion combinations of each gene were also constructed, including $\Delta katG1\Delta katG2$, $\Delta katB\Delta katG1$, $\Delta katB\Delta katG2$, $\Delta katB\Delta pgpD$, $\Delta katG1\Delta pgpD$, and $\Delta katG2\Delta pgpD$ to further elucidate the effects of each gene on ROS defense. All gene deletion mutants were constructed following previously described procedures (Toporek *et. al.*, 2019). Mutant genotypes were confirmed by PCR and sequencing of each PCR product.

Aerobic growth in minimal medium. All strains were activated from monoclonal colonies by inoculating in lysogeny broth (LB, 10 g L⁻¹ NaCl, 10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, Sigma-Aldrich) as a seed culture and incubated aerobically with shaking overnight at 30°C. Cultures were harvested by centrifugation at 10,000 x g, washed, and resuspended into minimal medium amended with 20mM lactate (20) All cultures were incubated aerobically at room temperature in 50mL of liquid medium in a 150mL serum bottle, which was continuously supplied with sterilized compressed air in the headspace and stirred at 300rpm until cultures reached stationary phase (35-60 hrs).

H₂O₂ scavenging assays. To compare the abilities of the *Shewanella* spp. to remove H₂O₂ from their environment, strains were seeded overnight at 30°C in lysogeny broth (LB, 10 g L⁻¹ NaCl, 10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, Sigma-Aldrich), harvested by centrifugation at 3000 rpm, washed and transferred into minimal medium amended with 20mM lactate at an OD₆₀₀ = 0.02. Cells were incubated at 30°C until harvesting at mid-log phase (OD₆₀₀ = 0.15-0.35), washed twice with minimal medium, then inoculated at OD₆₀₀ = 0.05 into a 24-well plate holding 2mL minimal medium amended with 20mM lactate and various concentrations of H₂O₂ (0-100 μ M). Samples were collected every 3-5 minutes and analyzed immediately for residual exogenous H₂O₂ using the resorufin-horseradish peroxidase colorimetric assay (21). Plates were incubated aerobically at room temperature with shaking for the duration of the experiments (30-200min). H₂O₂ scavenging rates followed exponential decay patterns, thus whole-cell scavenging data were treated as Michaelis-Menten single substrate enzyme kinetics for simplicity. Data were plotted in Lineweaver-Burk graphs (not shown), least squares regression was used to determine the best fit to find K_m and V_{max} values (where K_m is the Michaelis–Menten constant and V_{max} is the maximum reaction velocity), and Michaelis-Menten saturation plots were generated for each strain (Figs. 4b, 5b, 7b).

Microbially driven Fenton bioreactors with S. oneidensis MR-1 and single gene deletion mutants. Microbially driven Fenton bioreactors were run without contaminants as a pilot test to determine whether there was a difference in iron reduction ability or rates between wild-type *S. oneidensis* and single deletion mutant strains. Strains were activated from monoclonal colonies by inoculating in LB medium and incubated aerobically with shaking overnight at 30°C. Cultures were harvested by centrifugation at 13.2 rcf, washed, and resuspended into minimal medium amended with 20mM lactate overnight in 50mL tubes with no headspace to anaerobically precondition cells. *S. oneidensis* liquid cultures (10⁹ cells mL⁻¹ in M1 medium amended with 20mM lactate and 10 mM Fe(III) as ferric citrate) were inoculated in 100 mL of medium in 150-mL glass serum bottles. Anoxic conditions were maintained by injecting (hydrated) compressed nitrogen until the pool of Fe(III) was reduced to approximately 7 mM Fe(II) (approximately 7 h of anaerobic incubation; Fe(II)-generating phase). The compressed nitrogen line was then replaced by a hydrated compressed air line and the Fe(II)-containing *S. oneidensis* liquid culture was incubated under aerobic conditions until the 10 mM pool of Fe(II) was completely oxidized to

approximately 0-0.5 mM level (approximately 2.5 h of aerobic incubation; H₂O₂-generating phase). Reactor temperature (25°C) and pH (7.0) were held constant throughout all reactor phases.

5D. Results and Discussion.

In addition to the ROS stress caused by molecular oxygen oxidizing flavoenzymes, aerobic respiration is a main source of intracellular ROS due to incomplete reduction of O₂ to H₂O. Since the constructed mutants all lacked key enzymes related to oxidative stress protection, aerobic growth rates of each mutant strain were tested in minimal medium to determine effect, if any, of ROS-related gene deletion on cell growth. Single-deletion mutant strains $\Delta katB$ and $\Delta katG2$ grew to wild-type cell density (OD₆₀₀ = 1), but periplasmic glutathione peroxidase mutant $\Delta pgpD$ would only grow at 30°C (data not shown) and would not grow past OD₆₀₀ = 0.059 at room temperature (Fig. 3a). This diminished growth phenotype is consistent with a previous report that *pgpD* is the third most upregulated gene in *S. oneidensis* under H₂O₂ stress (Gao 2014). Without PgpD, cells may be unable to completely scavenge H₂O₂ at rates required for robust cell growth, and remain under chronic oxidative stress. Catalase-peroxidase mutant $\Delta katG1$ also displayed inhibited growth relative to wild-type and other mutants, but was still able to grow tenfold to OD₆₀₀ = 0.22 at room temperature. These data suggest PgpD and KatG-1 are likely key components of ROS detoxification during cell growth under the conditions tested.

Double mutant strains $\Delta katG1\Delta katG2$, $\Delta katB\Delta katG1$, and $\Delta katB\Delta pgpD$, grew to wild-type cell density OD₆₀₀ = 1 (Fig. 3b). $\Delta katG1\Delta pgpD$, $\Delta katG2\Delta pgpD$, and $\Delta katB\Delta katG2$ did not grow at room temperature, but maintained populations around their initial cell densities for the duration of the experiment at OD₆₀₀ = 0.017, 0.025, and 0.02, respectively (Fig. 3b).

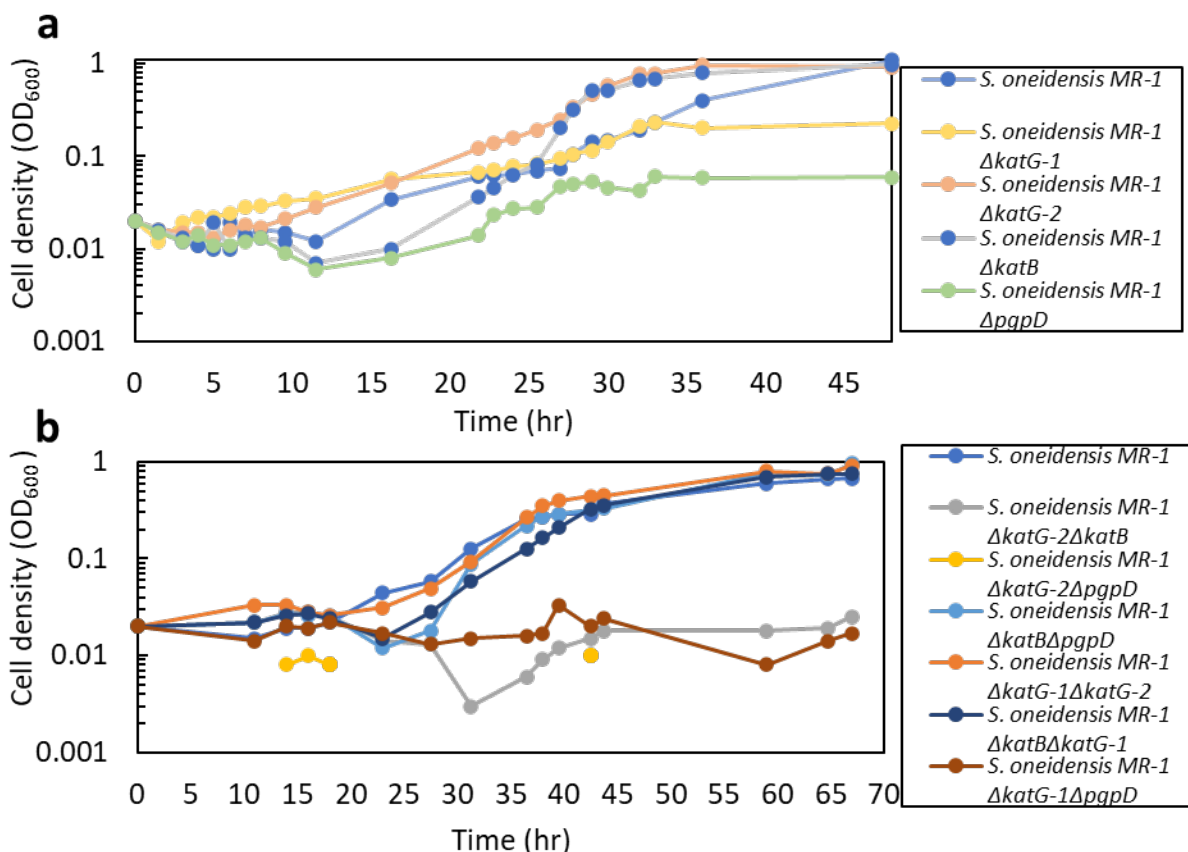
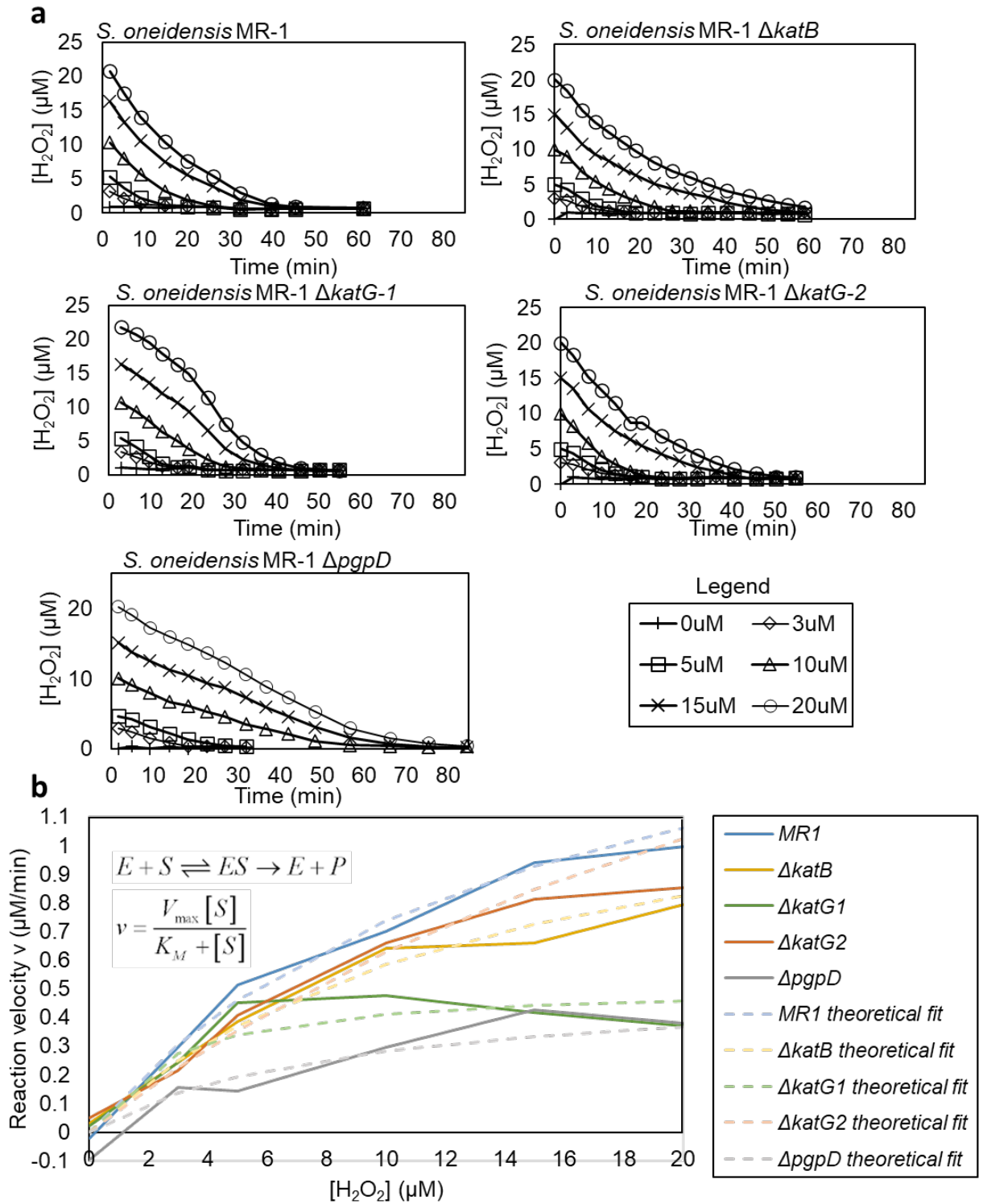


Figure 3. Aerobic growth curves of wild-type *S. oneidensis* MR-1 and ROS single- and double-deletion mutants in minimal medium. Broken lines indicate cell density is at 0.

All single mutants had impaired exogenous H_2O_2 scavenging rates when compared with the wild-type under the conditions tested (Fig. 4a), with $\Delta pgpD$ again displaying the lowest scavenging rate (Fig. 4b). $\Delta pgpD$ scavenged 20 μM exogenous H_2O_2 in 80 minutes, double the wild-type scavenging time, and displayed similarly depressed K_m and V_{max} when compared to wild-type (8.6 vs. 15.2 μM ; 0.5 vs 1.9 $\mu M/min$, respectively, Table 1). $\Delta katG1$ again showed the second most affected scavenging rate (K_m 2.7 μM , V_{max} 0.5 $\mu M/min$, Table 1) when compared with the wild type (Fig. 4), but the K_m is likely skewed low due to the mutant switching kinetic strategies during the experiment. $\Delta katG1$ appears to show zero order decay of H_2O_2 for the first 20 minutes of the experiment, and then displays first order decay for the duration of the experiment (Fig. 4a). $\Delta katG1$ may experience a 20-minute acclimation period to H_2O_2 , during which it may upregulate or induce other genes that are able to compensate for KatG1 and rapidly scavenge any remaining H_2O_2 . Surprisingly, $\Delta katB$ and $\Delta katG2$ showed near-wild-type patterns of H_2O_2 scavenging (K_m 13.8, 13.2 μM ; V_{max} 1.4, 1.5 $\mu M/min$, respectively) (Table 1). In another study, these genes were the most upregulated under exogenous H_2O_2 stress (16). It is likely that loss of either KatB or KatG2 within a background of other functional scavenging enzymes is not enough to show a significant deleterious effect during exogenous H_2O_2 scavenging.

Type	Strain	Deleted gene(s)	K _m (μM)	V _{max} (μM/min)
Wild-type	<i>S. oneidensis</i> MR-1	-	15.5	1.9
Single-deletion mutants	<i>S. oneidensis</i> MR-1 $\Delta katB$	catalase	13.8	1.4
	<i>S. oneidensis</i> MR-1 $\Delta katG1$	catalase-peroxidase HPI	2.7	0.5
	<i>S. oneidensis</i> MR-1 $\Delta katG2$	catalase-peroxidase HPII	13.2	1.5
	<i>S. oneidensis</i> MR-1 $\Delta pgpD$	periplasmic glutathione peroxidase	8.6	0.5
Double-deletion mutants	<i>S. oneidensis</i> MR-1 $\Delta katB \Delta katG1$	catalase, catalase-peroxidase HPI	6.4	1.0
	<i>S. oneidensis</i> MR-1 $\Delta katB \Delta katG2$	catalase, catalase-peroxidase HPII	2.4	0.4
	<i>S. oneidensis</i> MR-1 $\Delta katG1 \Delta katG2$	catalase-peroxidase HPI, HPII	4.9	1.0
	<i>S. oneidensis</i> MR-1 $\Delta katB \Delta pgpD$	catalase, periplasmic glutathione peroxidase	3.1	0.6
	<i>S. oneidensis</i> MR-1 $\Delta katG1 \Delta pgpD$	catalase-peroxidase HPI, periplasmic glutathione peroxidase	3.1	0.3
	<i>S. oneidensis</i> MR-1 $\Delta katG2 \Delta pgpD$	catalase-peroxidase HPII, periplasmic glutathione peroxidase	1.8	0.4
Wild-type	<i>S. oneidensis</i> MR-1	-	3.8	1.1
Wild-type	<i>S. algae</i> BrY	-	62.9	4.1
Wild-type	<i>S. baltica</i>	-	4195.2	320.3
Wild-type	<i>S. algae</i> MN-01	-	806.1	64.6

Table 1. Whole-cell exogenous hydrogen peroxide scavenging rates of assorted *Shewanella* strains and *S. oneidensis* gene deletion mutants.



Following these results, double mutants were tested to determine whether there was more significant impairment of H₂O₂ scavenging rates upon concurrent loss of multiple scavenging proteins (Fig. 5). $\Delta katB\Delta katG1$ and $\Delta katB\Delta katG2$ scavenging rates were significantly impaired (K_m 6.4, 2.4 μ M; V_{max} 1.0, 0.4 μ M/min, respectively) (Table 1) especially above 10 μ M H₂O₂. $\Delta katB\Delta pgpD$ and $\Delta katG1\Delta pgpD$ were also significantly impaired and took approximately twice as long as wild-type to clear exogenous H₂O₂ (K_m 3.1, 3.1 μ M; V_{max} 0.6, 0.3 μ M/min, respectively) (Table 1). $\Delta katG2\Delta pgpD$ was mildly impaired when compared to wild-type scavenging time, but significantly impaired at scavenging kinetics (K_m 1.8 μ M, V_{max} 0.4 μ M/min, Table 1). $\Delta katG1\Delta katG2$ scavenged H₂O₂ in a similar amount of time as wild-type (Fig. 5), but with different kinetics (K_m 4.9 μ M, V_{max} 1.0 μ M/min, Table 1).

Loss of only *katB* does not impact scavenging rates as much as loss of *katG1*, but when both *katB* and either *katG1* or *katG2* are absent from the genome, scavenging rates are severely affected. This suggests *S. oneidensis* likely utilize KatG1 or KatG2 in the absence of the main catalase KatB. Loss of PgpD in combination with loss of another scavenger also further impairs H₂O₂ scavenging rates, but not as severely.

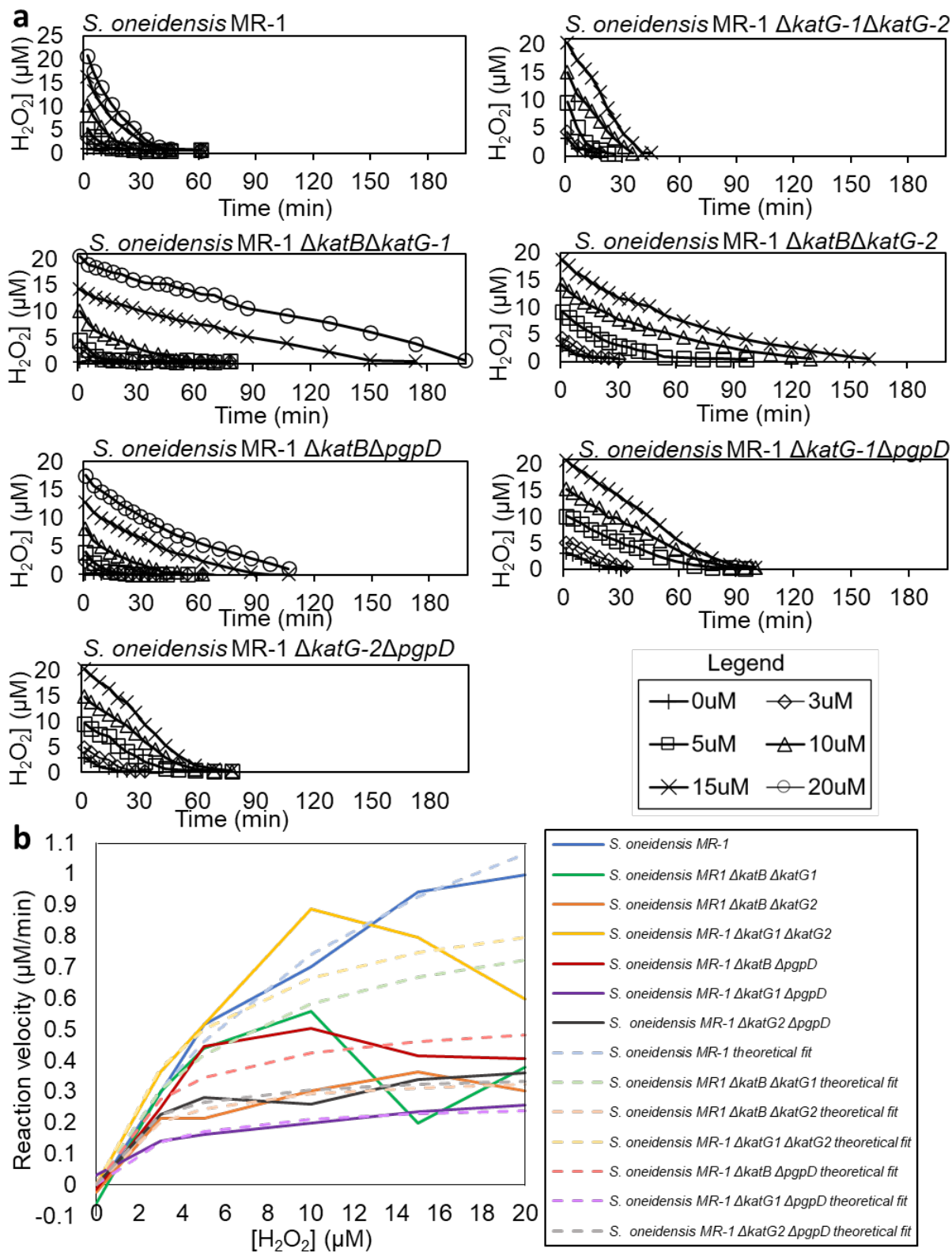


Figure 5. a. Whole-cell scavenging of exogenous H_2O_2 from 0-20 μM in minimal medium by wild-type *S. oneidensis* MR-1 and in-frame double gene deletion mutants *S. oneidensis* $\Delta katB \Delta katG-1$, $\Delta katB \Delta katG-$

2, $\Delta katG-1\Delta katG-2$, $\Delta katB\Delta pgpD$, $\Delta katG-1\Delta pgpD$, and $\Delta katG-2\Delta pgpD$. **b.** Michaelis -Menten saturation plots.

Under the conditions tested, all single mutant strains had similar rates of Fe(III) reduction (1mM/hr) and reoxidation (2.4mM/hr) (Fig. 6) with the wild-type strain. Clearly, mutants can still respire on Fe(III)-citrate at wild-type rates and this ability was not affected by lack of catalase or peroxidase. Double mutants were not tested. Incubations with a contaminant remains to be tested to determine whether mutants with reduced rates of H₂O₂ scavenging have enhanced 1,4-dioxane degradation rates.

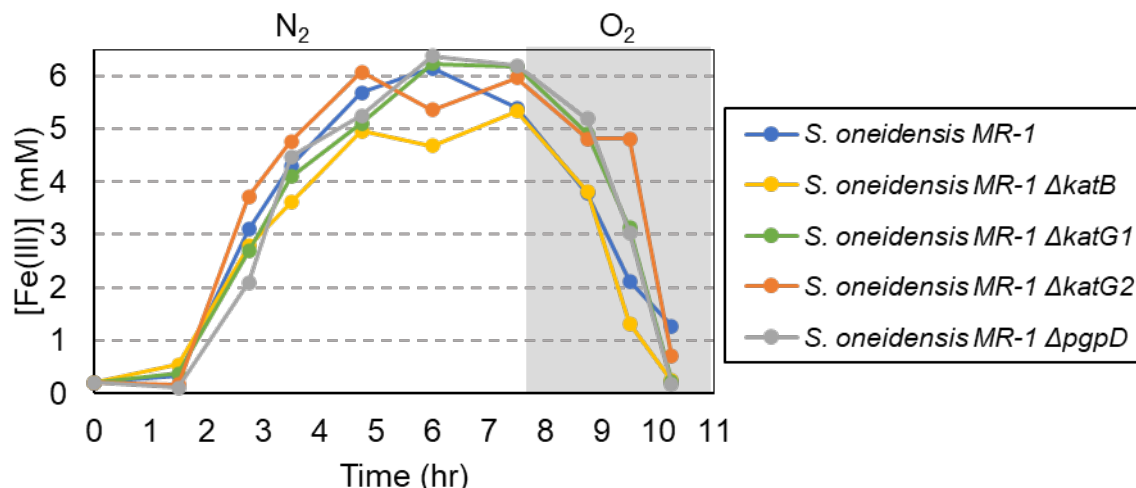


Figure 6. Microbially-driven Fenton reaction by wild-type *S. oneidensis* MR-1 and in-frame gene deletion mutants *S. oneidensis* $\Delta katB$, $\Delta katG-1$, $\Delta katG-2$, and $\Delta pgpD$. White or gray shading represents N₂ or compressed air injection, respectively, into bioreactor headspace.

Along similar lines, we tested other *Shewanella* species *S. algae* BrY, *S. baltica*, and *S. algae* MN-01 for their H₂O₂ scavenging rates, at a higher concentration range than previously (0-100 μ M) as a comparison (Fig. 7). *S. oneidensis* MR-1 was by far the weakest H₂O₂ scavenger tested (K_m 3.8 μ M, V_{max} 1.1 μ M/min, Table 1), and appears to reach saturation above 40 μ M, where the scavenging profile changes from zero order between 0-40 μ M to first order above 40 μ M. *S. baltica* and *S. algae* MN-01 were the strongest scavengers tested (K_m 4195.2, 806.1 μ M; V_{max} 320.3, 64.6 μ M/min, respectively) (Table 1) and likely were not saturated at conditions tested. Consequently, K_m and V_{max} reported here may be excessively high, and these experiments need to be repeated at a higher concentration range to determine true K_m and V_{max} . *S. algae* BrY (K_m 62.9 μ M, V_{max} 4.1 μ M/min, Table 1) placed in between *S. oneidensis* and the other two species for H₂O₂ scavenging rate.

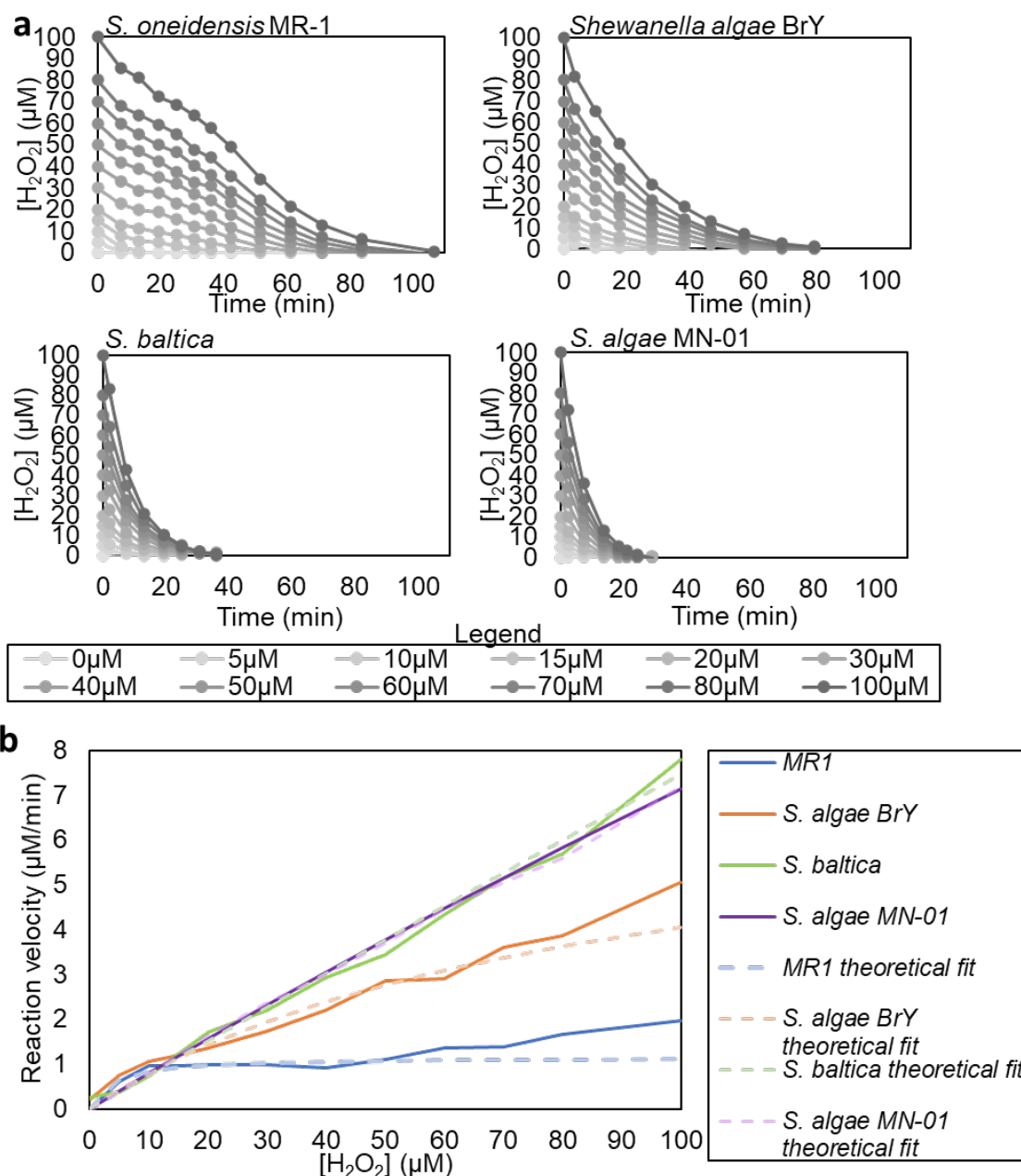


Figure 7. Whole-cell scavenging of exogenous H_2O_2 from 0-100 μM in minimal medium by wild-type *S. oneidensis* MR-1, *S. algae* BrY, *S. baltica*, and *S. algae* MN-01. **b.** Michaelis -Menten saturation plots.

5E. Conclusions and Future Implications. *S. oneidensis* single and double mutants lacking ROS scavenging genes all displayed impaired H_2O_2 scavenging abilities, yet retained wild-type Fe(III)-reduction rates. All mutants need to be tested in a microbially driven Fenton bioreactor with 1,4-dioxane, a contaminant previously degraded. Furthermore, comparison of *S. oneidensis* H_2O_2 scavenging with other *Shewanella* species demonstrates *S. oneidensis* as the weakest H_2O_2 scavenger tested. Perhaps the ability of *S. oneidensis* to degrade contaminants is due to such poor scavenging; if excessive H_2O_2 remains in the environment during the contaminant-degrading phase of the microbially driven Fenton reaction, there may be more availability for $\text{HO}^\bullet$ to attack and

degrade contaminants. The genomes of the *Shewanella* species tested have similar arsenals of ROS scavenging genes, yet dramatically different H₂O₂ scavenging rates. Additional Fe(III)-reducing *Shewanella* species should be tested for contaminant degradation ability and rates.

5F. References

1. McKinzi, A; DiChristina, TJ. Microbially Driven Fenton Reaction for Transformation of Pentachlorophenol. *Environ. Sci. and Technol.* 1999; 33(11) 1886-1891.
2. Sekar RS, DiChristina TJ. Microbially Driven Fenton Reaction for Degradation of the Widespread Environmental Contaminant 1,4-Dioxane. *Environ. Sci. and Technol.* 2014; 48: 12858-12867.
3. Sekar RS, DiChristina TD. Simultaneous Degradation of Comingled Contaminants Trichloroethylene, Tetrachloroethylene, and 1,4-Dioxane by a Microbially Driven Fenton Reaction. *Environ. Microbiol.* 2017; 82(21): 6335-6343.
4. Sekar RJ, DiChristina TJ. Degradation of the recalcitrant oil spill components anthracene and pyrene by a microbially driven Fenton reaction. *FEMS Microbiol. Lett.* 2017; 364 (21).
5. Imlay JA. Cellular defenses against superoxide and hydrogen peroxide. *Annu. Rev. Biochem.* 2008; 77:755–776.
6. Imlay, J. A. Where in the world do bacteria experience oxidative stress? *Environ. Microbiol.* 2019; 21(2), 521–530.
7. Seaver, L. C., & Imlay, J. A. Alkyl Hydroperoxide Reductase Is the Primary Scavenger of Endogenous Hydrogen Peroxide in *Escherichia coli*. *J. Bacteriol.* 2001; 183(24).
8. Khademan M, Imlay JA. *Escherichia coli* cytochrome *c* peroxidase is a respiratory oxidase that enables the use of hydrogen peroxide as a terminal electron acceptor. *Proc Natl Acad Sci USA.* 2017; 114(33):E6922-E6931.
9. Mishra, S., & Imlay, J. A. Why do bacteria use so many enzymes to scavenge hydrogen peroxide? *Arch. Biochem. Biophys.* 2012; 525(2), 145–160.
10. Messner, K. R., & Imlay, J. A. The identification of primary sites of superoxide and hydrogen peroxide formation in the aerobic respiratory chain and sulfite reductase complex of *Escherichia coli*. *J. Biol. Chem.* 199; 274(15), 10119–10128.
11. Messner, K. R., & Imlay, J. A. Mechanism of superoxide and hydrogen peroxide formation by fumarate reductase, succinate dehydrogenase, and aspartate oxidase. *J. Biol. Chem.* 2002; 277(45), 42563–42571.
12. Messner, K. R., & Imlay, J. A. In vitro quantitation of biological superoxide and hydrogen peroxide generation. *Meth. Enzymol.* 2002; 349(1969), 354–361.
13. Korshunov, S., & Imlay, J. A. Two sources of endogenous hydrogen peroxide in *Escherichia coli*. *Mol. Microbiol.* 2010; 75(6), 1389–1401.
14. Anjem, A., & Imlay, J. A. Mononuclear iron enzymes are primary targets of hydrogen peroxide stress. *J. Biol. Chem.* 2012; 287(19), 15544–15556.
15. Li N, Luo Q, Jiang Y, Wu G, Gao H. Managing oxidative stresses in *Shewanella oneidensis*: intertwined roles of the OxyR and OhrR regulons. *Environ. Microbiol.* 2014; 16(6):1821-34.
16. Jiang Y, Dong Y, Luo Q, Li N, Wu G, Gao H. Protection from oxidative stress relies mainly on derepression of OxyR-dependent KatB and Dps in *Shewanella oneidensis*. *J Bacteriol.* 2014;196(2):445-58. doi: 10.1128/JB.01077-13..

17. Ghosal D, Omelchenko MV, Gaidamakova EK, Matrosova VY, Vasilenko A, Venkateswaran A, Zhai M, Kostandarithes HM, Brim H, Makarova KS, Wackett LP, Fredrickson JK, Daly MJ. How radiation kills cells: survival of *Deinococcus radiodurans* and *Shewanella oneidensis* under oxidative stress. FEMS Microbiol. Rev. 2005; 29:361–375.
18. Qiu X, Sundin GW, Wu L, Zhou J, Tiedje JM. Comparative analysis of differentially expressed genes in *Shewanella oneidensis* MR-1 following exposure to UVC, UVB, and UVA radiation. J. Bacteriol. 2005; 187: 3556–3564.
19. Qiu X, Daly MJ, Vasilenko A, Omelchenko MV, Gaidamakova EK, Wu L, Zhou J, Sundin GW, Tiedje JM. Transcriptome analysis applied to survival of *Shewanella oneidensis* MR-1 exposed to ionizing radiation. J. Bacteriol. 2006; 188:1199–1204.
20. Myers CR, Nealson KH. Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. Science. 1988; 240(4857):1319-21.
21. Zhou M, Diwu Z, Panchuk-Voloshina N, Haugland RP. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. Anal Biochem. 1997; 253(2):162-8.

Appendix A. List of Scientific/Technical Publications.

Articles

1. Sekar RS, DiChristina TJ. Microbially Driven Fenton Reaction for Degradation of the Widespread Environmental Contaminant 1,4-Dioxane. *Environ. Sci. and Technol.* 2014; 48: 12858-12867.
2. Sekar RS, DiChristina TJ. Simultaneous Degradation of Comingled Contaminants Trichloroethylene, Tetrachloroethylene, and 1,4-Dioxane by a Microbially Driven Fenton Reaction. *Environ. Microbiol.* 2017; 82(21): 6335-6343.
3. Toporek YJ, Shin HD, DiChristina TJ. The microbially-driven Fenton reaction does not degrade the recalcitrant environmental pollutant perfluorooctanoic acid. *Manuscript submitted to FEMS Microbiology Letters.*
4. Xie N, Toporek Y, DiChristina TJ, Taillefert M. "Impact of Orthophosphate on the Dark Production of H₂O₂ during Microbially-driven Fenton Reactions with Iron(III) Oxides." *Manuscript submitted to Environmental Science & Technology.*

Conference Proceedings

Nan Xie, Martial Taillefert. "Development of a kinetic model to investigate the mechanism of dark ROS production during microbially-driven Fenton reactions". *Session: Kinetic Studies & Mathematical Models to Investigate Biogeochemical Reaction Mechanisms in Complex Systems*. American Chemical Society (ACS) Annual Meeting, Online Oral Presentation (2020/08)

Yael Toporek, Nan Xie, **Thomas J DiChristina**, Martial Taillefert. Novel Microbially-Driven Fenton Reaction for In Situ Remediation of Groundwater Contaminated with 1,4-dioxane, Tetrachloroethene (PCE) and Trichloroethene (TCE). Poster Presentation, SERDP and ESTCP Symposium, Washington, DC (2019/11)

Nan Xie, Yael Toporek, Thomas J. DiChristina, and Martial Taillefert. "Effect of secondary mineralization of ferrihydrite on dark production of ROS via microbially-driven Fenton reaction". *Session: Sorption & Redox at Mineral-Water Interfaces & the Impact on the Biogeochemical Cycling of Trace & Major Elements*. American Chemical Society (ACS) Annual Meeting, Oral Presentation San Diego, CA (2019/08)

Yael Toporek, Thomas J DiChristina. Enhancement of the microbially-driven Fenton reaction via genetic manipulation of *Shewanella* genes involved in oxidative stress response. Poster Presentation, Applied and Environmental Science Track, American Society for Microbiology (ASM) Microbe. Atlanta, GA (2019/06)

Yael Toporek, Nan Xie, Thomas J DiChristina, Martial Taillefert. Degradation of 1,4-dioxane by a Microbially Driven Fenton Reaction Operated in a Flow-Through Reactor Configuration. Poster Presentation, SERDP and ESTCP Symposium, Washington, DC (2018/11)

Yael Toporek, Nan Xie, Thomas J DiChristina, Martial Taillefert. Simultaneous degradation of comingled contaminants by a microbially-driven Fenton reaction operated in fed-batch and flow-

through reactor modes. Poster Presentation, Applied and Environmental Science Track, American Society for Microbiology (ASM) Microbe. San Francisco, CA (2018/06)

Nan Xie, Yael Toporek, Thomas J. DiChristina, and Martial Taillefert. “Using Flow-through Columns to Optimize the Microbially-driven Fenton Reaction for the in-situ Degradation of Organic Contaminants”. *Session: Contaminated Site Remediation through Microbial, Geological & Chemical Processes*. American Chemical Society (ACS) Annual Meeting, Oral Presentation, New Orleans, LA (2018/03)

Yael Toporek, Nan Xie, Thomas J DiChristina, Martial Taillefert. Simultaneous degradation of commingled contaminants by a microbially-driven Fenton reaction operated in fed-batch and flow-through reactor configurations. Poster Presentation, Eleventh (11th) International Conference on the Remediation of Chlorinated and Recalcitrant Compounds. Palm Springs, CA (2018/04)

Yael Toporek, Nan Xie, Thomas J DiChristina, Martial Taillefert. Simultaneous degradation of commingled contaminants by a microbially-driven Fenton reaction operated in fed-batch and flow-through reactor configurations. Poster Presentation, SERDP and ESTCP Symposium, Washington, DC (2017/11)

Yael Toporek, Ramanan Sekar, Nan Xie, Thomas J DiChristina, Martial Taillefert. Microbially-driven Fenton reaction for degradation of chlorinated solvents and oil spill contaminants. Poster Presentation, Applied and Environmental Science Track, American Society for Microbiology (ASM) Microbe. New Orleans, LA (2017/06)

Yael Toporek, Ramanan Sekar, Nan Xie, Thomas J DiChristina, Martial Taillefert. Microbially-driven Fenton reaction for degradation of oil spill contaminants. Poster and Oral Presentation, Fourth (4th) International Symposium on Bioremediation and Sustainable Environmental Technologies, Miami, FL (2017/05)

Nan Xie, Yael Toporek, Thomas J. DiChristina, and Martial Taillefert. “Potential for Microbially-driven Fenton Reaction in Subsurface Environments”, Poster Presentation, 4th Annual Southeast Biogeochemistry Symposium. Athens, GA (2017/03)

Book Chapters

Szeinbaum, N., Toporek, Y.J., Shin, H.D., and DiChristina, T.J. (2018). Application of *Shewanella* to water treatment issues. April 2020. *Encyclopedia of Water, Science, and Technology*. ISBN: 978-1-119-30075-5