Effect of O$_2$ exposure on perchlorate reduction by *Dechlorosoma* sp. KJ

Yanguang Song, Bruce E. Logan*

Department of Civil and Environmental Engineering, The Pennsylvania State University, 212 Sackett Bldg., University Park, PA 16802, USA

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Abstract

Anaerobic bioreactors have been developed to remove perchlorate from water, but backwashing and operational interruptions can expose biofilms to oxygen. While it is well known that oxygen is a preferential electron acceptor to perchlorate for perchlorate-respiring bacteria, little is known about the effect of oxygen exposure or redox potentials on perchlorate reduction. Four different dissolved oxygen scavengers were tested for their ability to quickly restore anaerobic conditions and allow perchlorate reduction by a facultative, perchlorate respiring bacterium *Dechlorosoma* sp. KJ. Of the four different oxygen scavengers tested (Oxyrase™, L-cysteine, Na$_2$S and FeS), only Oxyrase™ was able to rapidly (<30 min) scavenge dissolved oxygen and allow cell growth. There was no cell growth after addition of Na$_2$S and FeS, and L-cysteine produced a long lag in cell growth. To investigate the effect of dissolved oxygen on perchlorate reduction, anaerobically grown cultures *Dechlorosoma* sp. KJ, were exposed to dissolved oxygen for various periods ranging from 1 to 32 h. Perchlorate reduction and redox potential were then measured for cells returned to an anaerobic environment containing an oxygen scavenger. It was determined that cells exposed to dissolved oxygen for more than 12 h were incapable of reducing perchlorate. Cells exposed to dissolved oxygen for less than 12 h quickly reduced the redox potential to negative values (−127 mV to −337 mV) and were able to reduce perchlorate or chlorite. Our results suggest that aeration during backwashing of biofilm reactors, or exposure of perchlorate-degrading cell suspensions to dissolve oxygen for less than 12 h, will not be detrimental to the ability of perchlorate-degrading bacteria to use perchlorate as an electron acceptor.

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*Corresponding author. School of Civil Engineering and Geosciences, University of Newcastle Upon Tyne, Geosciences, Cassie Building, Newcastle Upon Tyne NE1 7RU, UK. Tel.: +44-814-863-7908; fax: +44-814-863-7304.

E-mail address: blogan@psu.edu (B.E. Logan).

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1. Introduction

Perchlorate has been found in ground waters in the United States, at typical concentrations of 50–200 µg/L, primarily as a result of its production and use in solid rocket propellant [1]. The US EPA has identified perchlorate users and manufacturers in 44 states, and perchlorate releases in at least 20 states [2]. Perchlorate contamination poses a significant health threat to humans because even at low concentrations perchlorate is known to affect the function of the thyroid gland by inhibiting the uptake of iodide and affecting hormone production [2–4]. Perchlorate releases are estimated to have affected the drinking water of 15 million people that reside mostly in the states of California, Nevada, Utah, and Arizona. The US EPA proposed a draft reference dose of 0.03 µg per kg of body weight per day, which could lead to a drinking water standard of 1 µg/L to protect human health [2]. Based on this information, the California Department of Health Service in
California decreased the action level of perchlorate in drinking water in California from 18 μg/L to the detection limit of ion chromatograph, 4 μg/L [5].

Recent studies have demonstrated the ubiquity and diversity of perchlorate-respiring bacteria (PRB) that can couple growth to the reduction of perchlorate under anaerobic conditions [6–13]. Analysis of the 16S rRNA sequences of these strains has shown that all were members of the Proteobacteria and that a majority of these PRB were located in the β-subclass [14]. Perchlorate is degraded via a three-step process of: ClO$_3^- \rightarrow$ ClO$_2^- \rightarrow$ O$_2^- + Cl^-$, and the molecular oxygen evolved is immediately used for biomass generation [13]. Although a single enzyme (perchlorate reductase) has been found that can catalyze both chlorate- and perchlorate-reduction [15], not all chlorate-respiring bacteria are capable of respiration with perchlorate [7]. Thus, it may be that another enzyme is involved in perchlorate reduction in a single organism or that different forms of the enzyme are inactive with some bacteria. The last step of chlorite disproportionation does not yield any energy for the cell and is catalyzed by an enzyme (chlorite dismutase) only recently isolated [16,17].

Bioreactors have been developed to remove perchlorate from drinking water sources and wastewaters [18–20]. PRB can be exposed to dissolved oxygen in different ways in these reactors, but the effects of oxygen exposure on perchlorate reduction are not well understood. It is well known that oxygen is a preferential electron acceptor to perchlorate by PRB, and that high dissolved oxygen concentrations completely inhibit perchlorate reduction [15,21]. However, while oxygen is released during perchlorate degradation it is rapidly used by PRB and does not accumulate to high concentrations in solution during perchlorate degradation. Operation of fixed bed reactors can result in instances where PRB are exposed to high concentrations of dissolved oxygen [22]. For example, periodic backwashing of fixed bed systems must be performed to limit the growth of PRB bacteria and prevent clogging. Air sparging enhances the efficiency of backwashing, but exposure of PRB to oxygen for long periods could inhibit perchlorate reduction when the system is returned to service. Alternatively, a temporary reactor shut down for servicing could also expose the biofilm to high concentrations of dissolved oxygen.

The effects on perchlorate reduction of long-term exposure of PRB to high concentrations of dissolved oxygen has only been indirectly studied. Attaway and Smith [21] found that addition of air to the microbial community during perchlorate reduction immediately terminated the process and aeration for 12 h permanently destroyed the ability of the culture to reduce perchlorate. However, the applicability of this finding to other PRB is not known as most PRB are facultative anaerobes, but the PRB used in their study (Wolinella succinogenes HAP-1) [8] is only aero-tolerant and not facultative. The effect of dissolved oxygen on two key enzymes involved in perchlorate reduction is known, but cannot explain Attaway et al.’s findings. Kengen et al. [15] reported that the half time of inactivity of perchlorate reductase following exposure to oxygen was about 2–3 days, not hours, as found for the PRB HAP-1. Chlorite dismutase activity is unaffected by dissolved oxygen [13].

In order to better understand the effect of dissolved oxygen on perchlorate reduction, we examined the effect on perchlorate reduction of exposure of perchlorate-degrading cultures of the PRB Dechlorosoma sp. KJ to dissolved oxygen for periods ranging from 1 to 32 h. While the redox potentials needed for denitrifying and methanogenic cultures are well known, they have not been previously reported for PRB. We therefore tested four different reducing agents for their ability to scavenge oxygen and provide a low redox potential for cell growth. Using one of these oxygen scavengers, we then monitored the redox potential and perchlorate reduction of anaerobic cell suspensions exposed to oxygen for various time periods that were returned to anaerobic conditions.

2. Material and methods

2.1. Bacterium and media

Dechlorosoma sp. KJ, originally isolated from a perchlorate-degrading packed bed bioreactor [7], was used in all experiments unless stated otherwise. Media were prepared using ultrapure water (Milli-Q system; Millipore Corp., New Bedford, MA) and research-grade chemicals in the amounts (per liter) indicated below. The basal medium contained: 1.55 g K$_2$HPO$_4$, 3H$_2$O, 0.85 g NaH$_2$PO$_4$, 2H$_2$O, 0.5 g NH$_4$H$_2$PO$_4$, 50 mg MgSO$_4$·7H$_2$O, 3 mg EDTA, 2 mg ZnSO$_4$·7H$_2$O, 1 mg CaCl$_2$·2H$_2$O, 4 mg FeSO$_4$·7H$_2$O, 0.4 mg Na$_2$MoO$_4$·2H$_2$O, 0.2 mg CuSO$_4$·5H$_2$O, 0.4 mg CoCl$_2$·6H$_2$O, 1 mg MnCl$_2$·4H$_2$O, 0.1 mg NiCl$_2$·6H$_2$O, 0.15 mg Na$_2$SeO$_3$ and 0.6 mg H$_3$BO$_3$ [7,13]. Unless stated otherwise, acetate (C$_2$H$_3$O$_2$, 2 g/L) and perchlorate (ClO$_4^-$, 0.5 g/L) salts were added as the electron donor and acceptor, respectively. The pH of the media was adjusted to 7.0 using NaOH. Anaerobic medium was sterilized by autoclaving and degassed in an anaerobic glove box (Coy Scientific Products, Grass Lake, MI).

2.2. Preparation of cell suspensions

Anaerobically grown cell suspensions of strain KJ were used in all oxygen scavengers and oxygen exposure experiments unless stated otherwise. Cells were
harvested during late-log-phase growth (optical density at 600 nm (OD\textsubscript{600}) of 0.3–0.4), washed once at 5000 × g for 10 min, and resuspended in medium to the same OD\textsubscript{600} in the glove box.

### 2.3. Oxygen scavengers

Four different reducing agents were tested for their ability to scavenge dissolved oxygen from the media while allowing bacterial growth: Oxyrase\textsuperscript{TM}, l-cysteine · HCl [23], Na\textsubscript{2}S [24] and FeS [24]. Chemicals were added from sterile anoxic aqueous stock solutions to the medium at concentrations typically used or recommended for these compounds: Oxyrase\textsuperscript{TM}, 0.02 mL/mL; l-cysteine · HCl, 0.5 mg/mL; Na\textsubscript{2}S, 20 mg/L; and FeS, 0.07 mL-precipitant/mL (added in excess).

### 2.4. Dissolved oxygen probe measurements of oxygen removal rates

Oxygen removal rates of the four different oxygen scavengers were examined in abiotic growth medium by using an oxygen probe (YSI Model 5331, YSI Incorporated, Yellow Springs, OH) and monitor (YSI Model 5300). The probe was calibrated with air-saturated water at 25°C before each experiment. Each oxygen scavenger was added at the final concentration as above to a 20-mL chamber containing 3 mL of air-saturated abiotic growth medium, and constantly stirred. A redox potential indicator, resazurin (\(E_0 = -51\) mV (pH 7.00)) [25], was added at a final concentration of 0.5 mg/L. Oxygen removal rates were calculated from the slope of the dissolved oxygen concentration change over time.

### 2.5. Effect of different oxygen scavengers on cell growth

Each oxygen scavenger was added at the final concentration as above to 130 mL serum bottles prepared under anaerobic conditions containing 95 mL of medium and 5 mL of cell suspension (1.67 × 10\textsuperscript{9} cells/mL). The serum bottles were fitted with butyl rubber stoppers and aluminum crimp seals, and incubated at 25°C. The effect of the oxygen scavengers was monitored on cell growth and perchlorate reduction. Resazurin was added at a final concentration of 0.5 mg/L.

### 2.6. \(O_2\) exposure procedures

A cell suspension (75 mL) was transferred to 2000-mL flask prepared under anaerobic conditions containing 1425 mL of medium. After incubating 20 h at 25°C in the anaerobic glove box, the cell suspension (150 mL) was split and placed into ten separate flasks (200 mL each). One flask served as an anaerobic control (ANC), and was kept in the anaerobic glove box for the duration of the experiment. Eight flasks were taken out of the glove box, aerated for a different period of time (1, 2, 4, 8, 12, 22, 28, and 32 h), and then returned back to the glove box. Oxyrase\textsuperscript{TM} (0.02 mL/mL) was added to each flask when it was returned to the glove box in order to scavenge any remaining dissolved oxygen. One flask (aerobic control (AC)) was aerated continuously for the duration of the experiment. The effect of aeration was monitored on cell growth and perchlorate reduction. Following the return of samples to the glove box redox potentials (\(E_0\), potential relative to the normal hydrogen electrode) were measured with two platinum redox electrodes (96-78-BN; ThermoOrion, Beverly, MA) filled with 4 M KCl saturated with Ag/AgCl.

### 2.7. Analytical techniques

Cell suspensions were monitored by optical density at 600 nm (OD\textsubscript{600}). Concentrations of perchlorate, acetate, and chloride anions were determined with an ion chromatograph (DX500; Dionex, Sunnyvale, CA) equipped with an AS11 column and guard column, a self-regenerating suppressor, and an autosampler. Perchlorate ion was measured using 100 mM NaOH eluent. For acetate and chloride measurements, a 10 mM NaOH eluent was used. The minimum detection limit for perchlorate was 4 \(\mu\)g/L. Dissolved oxygen concentrations were measured using a YSI Model 5331 Standard Oxygen probe with a YSI Model 5300 biological oxygen monitor (YSI Incorporated, Yellow Springs, OH).

### 3. Results

#### 3.1. Oxygen scavengers

All four oxygen scavengers tested removed over 50% of the dissolved oxygen in 30 min (Fig. 1). FeS removed dissolved oxygen the most rapidly, while Oxyrase\textsuperscript{TM}
reduced the dissolved oxygen the most slowly. In tests with bacteria, however, only Oxyrase™ did not adversely affect cell growth on perchlorate. The rate of perchlorate reduction and cell growth was more rapid with Oxyrase™ than with FeS, Na₂S or L-cysteine. The growth yield using Oxyrase™ was larger than the control (Fig. 2), suggesting that some of the proprietary ingredients in Oxyrase™ were used as a substrate for cell growth. Cells grown with L-cysteine exhibited a long lag phase (25 h) prior to cell growth. Although FeS and Na₂S removed the dissolved oxygen in the abiotic medium more rapidly than the other two scavengers (Fig. 1), there was no appreciable cell growth or perchlorate reduction in the presence of these two chemicals for 60 h (Fig. 2). Cells grown with FeS and Na₂S eventually (> 60 h) removed all perchlorate in the medium (data not shown). There was no perchlorate reduction in abiotic controls of oxygen scavengers.

3.2. Redox potential

The redox potential in abiotic tests (Fig. 1) was below −110 mV for all oxygen scavengers except Oxyrase™ as indicated by the color change of resazurin from pink to clear. In the absence of bacteria, Oxyrase™ reduced the redox potential to only 277 mV. In tests where bacteria respired perchlorate (Oxyrase™, L-cysteine, and the control; Fig. 2), the redox potential remained above –110 mV as the color of the tubes containing resazurin remained pink. A positive redox potential was confirmed by the presence of ca. 1.6 mg/L of dissolved oxygen in all actively growing samples. Changes in redox potential during cell growth on perchlorate were further examined in tests described below.

3.3. Effect of oxygen exposure on redox potential change

Anaerobically growing cells exposed to oxygen were capable of rapidly reducing the redox potential to a low level (< −127 mV) after being exposed to oxygen for 8 h (T8, Fig. 3). The redox potential of this suspension exposed to dissolved oxygen for 8 h was carefully monitored following exposure to aerobic conditions. There was a rapid drop in redox potential observed just prior to rapid perchlorate reduction, followed by a gradual increase in redox potential due to oxygen evolution after the culture began rapidly reducing perchlorate (Fig. 3).

The rapid drop in redox potential was always observed in other experiments when the cells were returned to anaerobic conditions following exposure to dissolved oxygen for up to 12 h (Fig. 4). During aeration, dissolved oxygen concentrations were about 6–7 mg/L. The final dissolved oxygen concentration of most samples (except the AC) containing Oxyrase™ was below 0.08 mg/L. The initial redox potential (E₀) of the cell suspension growing anaerobically in degassed medium in the glove box was −327 mV. This redox potential increased to 420 mV when samples were removed from the glove box and aerated (Fig. 4). Cells
returned to the glove box after exposure to dissolved oxygen for less than 12 h were able to rapidly decrease the redox potential after being returned to the glove box. For example, when cells were exposed to dissolved oxygen for 1 h (T1), and then were returned to the anaerobic glove box and amended with Oxyrase™, the redox potential rapidly (<30 min) decreased to −337 mV. Similarly, other samples returned to the glove box within 12 h (T2, T4 and T8), exhibited a rapid decrease in redox potential as well. The most negative redox values achieved by these samples seemed to decrease (−333, −315 and −127 mV for T2, T4 and T8, respectively) with the extent of exposure to dissolved oxygen (Fig. 4). For the sample exposed to oxygen for 12 h (T12), a negative redox potential was never reached, and after 60 min the redox potential began to increase. Samples exposed to oxygen for periods longer than 12 h (T22, T28 and T32), had redox potentials (281, 295 and 282 mV; Fig. 4) similar to that produced solely by the Oxyrase™ (277 mV).

3.4. Effect of oxygen exposure on perchlorate reduction

Growing cells exposed to dissolved oxygen for up to 8 h retained the ability to rapidly reduce perchlorate (Fig. 5). This exposure time of 8 h was shown above to be critical for the cells to resume a negative redox potential (T8, Fig. 4). The rate at which perchlorate could be degraded was inversely proportional to time of exposure to dissolved oxygen up to 12 h. Aeration for 12 or more hours destroyed the ability of the culture to completely reduce perchlorate, even though a low redox potential was rapidly restored by the addition of Oxyrase™ (Fig. 4). With aeration for more than 22 h, the cultures completely lost the capability to reduce perchlorate for at least 10 days (data not shown). The final acetate concentrations of most samples (except the AC and the ANC) containing Oxyrase™ was above 600 mg/L. The washed cells of the culture exposed to oxygen for 12 h or more eventually grew using perchlorate, but only after 3–7 days of incubation. In general, the incubation time needed to remove perchlorate increased with the time that the sample had been aerated.

4. Discussion

Oxygen scavengers such as L-cysteine are routinely used to maintain strict anaerobic conditions for methanogenic cultures under laboratory conditions, but they have not been previously tested or reported for use in maintaining anaerobic conditions for perchlorate reduction. Of the four oxygen scavengers tested, Oxyrase™ appeared to be the most useful oxygen scavenger for growing PRB. L-cysteine produced long lags in growth, and PRB did not grow in cultures containing FeS and Na2S. Exposure of cultures growing anaerobically, and using perchlorate, to dissolved oxygen (DO) for 12 h or more destroyed the capability of these bacteria to reduce perchlorate even when they were returned to an anaerobic environment, and the remaining DO was scavenged using Oxyrase™ (Fig. 5). This exposure time is consistent with that found by others. Attaway and Smith [21] found that exposure of perchlorate respiring culture containing primarily the aero-tolerant bacterium W. succinogenes HAP1 [8] to DO for 12 h terminated the ability of the cell to reduce perchlorate. However, the short time needed to inhibit perchlorate reduction is inconsistent with an explanation that oxygen affected key enzymes known to use for perchlorate reduction. Perchlorate reductase activity is oxygen sensitive, but it has a half time of inactivation of 2–3 days [15], which is
much larger than the 12 h observed here. Chlorite dismutase activity is not affected by dissolved oxygen [9,16,17], consistent with other tests in our laboratory showing that cells retained chlorite dismutase activity when the cells were exposed to dissolved oxygen for 12 h (data not shown). Thus, the lack of perchlorate reduction by the cells exposed to high dissolved oxygen concentrations for 12 or more hours does not appear to be due to the effect of oxygen on either perchlorate reductase or chlorite dismutase.

Cells were only capable of resuming perchlorate reduction after exposure to dissolved oxygen when they were able to rapidly reduce the redox potential to negative values. Thus, there must be a link between the inability of the cells to reduce perchlorate and the ability to create this negative redox potential. When anaerobically growing cells were exposed to dissolved oxygen, perchlorate reduction resumed only when cells reduced the redox potential to less than –127 mV in the presence of Oxyrase™ (Fig. 4). This large initial decrease in redox potential for the cells able to degrade perchlorate can not be attributed solely to Oxyrase™, as this chemical reduced the redox potential to only 277 mV in an abiotic medium. The ability to produce a redox potential of less than –127 mV must therefore due to microbial activity.

The lack of an effect of dissolved oxygen on the two enzymes known to be involved with perchlorate reduction (perchlorate reductase and chlorite dismutase), suggest that there must be at least one oxygen-sensitive enzyme involved in perchlorate respiration reduction. The activity of this enzyme would function to sharply reduce the redox potential of the medium when cells were first returned to anaerobic conditions. A logical candidate for this unknown oxygen-sensitive enzyme would be a high-affinity terminal oxidase [26,27]. Other bacteria, such as E. coli, have branched electron transport pathways that lead to different terminal oxidases. In E. coli, cytochrome bd complex has a high affinity for oxygen and is the dominant oxidase under low oxygen tension [28,29], while the cytochrome bo complex has a low affinity for oxygen and is predominant under high tension. Thus, it is possible that there are two different terminal oxidases expressed by PRB under different oxygen levels, and that perchlorate reduction is coupled with the activity of a low oxygen tension cytochrome. The ability of cells to reduce the redox potential to negative values demonstrated the presence and activity of a low oxygen tension enzyme. When perchlorate reduction was lost (between 8 and 12 h), this high-affinity terminal oxidase was either blocked or became damaged by exposure to high oxygen concentrations (Fig. 4).

These results suggest that the ability of bacteria to reduce perchlorate, which produces dissolved oxygen from chlorite, can occur as long as low oxygen tensions are maintained. However, exposure of cells to high DO concentrations for 12 or more hours will inhibit perchlorate reduction likely due to the loss of some key respiratory enzyme that has not yet been isolated from PRB. The identification and isolation of this low-oxygen tension respiratory enzyme are interesting areas for future research, and may lead to new clues for understanding the ability of bacteria to respire using perchlorate.

5. Conclusions

The following conclusions can be made regarding the effect of oxygen exposure on perchlorate reduction by Dechlorosoma sp. KJ:

a. Oxyrase™ can be used in laboratory experiments with perchlorate respiring bacteria to reduce oxygen to low levels (~1.6 mg/L).

b. At the onset of perchlorate reduction by Dechlorosoma sp. KJ, the redox potential was initially reduced to a negative value (~127 to –333 mV), but it increased as DO was evolved during perchlorate reduction.

c. PRB growing anaerobically that were exposed to high DO levels retained the ability to rapidly reduce perchlorate for up to 8 h, but could not reduce perchlorate after 12 h.

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References


