FINAL REPORT

An Enzymatic Bioassay for Perchlorate

SERDP Project ER-1530

JULY 2010

John D. Coates
Mark Heinnickel
University of California Berkeley

Laurie A. Achenbach
Southern Illinois University

SERDP
DOD · EPA · DOE

Table of Contents

Abstract

We developed a simple robust perchlorate-specific colorimetric bioassay under SERDP Project 1530 "An Enzymatic Bioassay for Perchlorate". Ammonium perchlorate represents 90% of all perchlorate salts manufactured and is used as an energetics booster or oxidant in solid rocket fuels. Its presence in the environment from legal historical discharge poses a significant health threat. Current analytical technologies for the identification and quantification of perchlorate at low levels are based on ion chromatography with conductivity or mass spectrometry detection. Although sensitive, these methods are inefficient being slow and arduous, exceptionally expensive, and require significant sample preparation by highly trained personnel in specialized laboratories. As such, their application for the rapid delineation of contamination zones in an environment is neither time nor cost effective. The technology outlined here offers a rapid, sensitive, specific, and cost effective solution that can be performed onsite with minimal training utilizing robust ubiquitous laboratory equipment.

Figure 1. Schematic of the steps involved in the developed perchlorate specific bioassay.

Technology description.

The developed bioassay (Fig. 1) uses the partially purified perchlorate reductase (PCR) enzyme from *Dechloromonas agitata* to detect perchlorate with the redox active dye phenazine methosulfate (PMS) and nicotine adenine dinucleotide (NADH). By using a specific addition scheme and covering all reactions with mineral oil, the reaction can be performed on the benchtop with a lower detection limit of 2 ppb when combined with perchlorate purification and concentration by solid phase extraction (SPE). We have accurately analyzed perchlorate concentrations (0-17,000ppb) using the bioassay in the presence of a range of ions (nitrate, phosphate, sulfate, iron, chloride) at a concentration of 100 ppm.

Expected benefits.

The technology outlined here offers a rapid, sensitive, specific, and cost effective solution that can be performed onsite with minimal training utilizing robust ubiquitous laboratory equipment. The cost of an ion chromatography system with conductivity detection can reach $50,000 US dollars ($500,000 with mass spectrometry detection), and has a consumables charge of >$1 per sample, the outlined bioassay has a much lower instrument and materials cost (a hand-held spectrophotometer ~$300, reusable SPE columns $250) with consumables of approximately $0.13 per sample. Furthermore, in contrast to an ion chromatograph the bioassay allows for multiple samples to be assayed simultaneously while achieving a minimum detection limit of 2 ppb.

Introduction.

Proposed project objective:

The objective of the studies was to develop a highly sensitive and specific analytical colorimetric assay for the rapid determination of perchlorate in environmental samples. In our original project description this objective was to be achieved by combining a previously developed colorimetric enzymatic assay for chlorite (ClO_2^-) with the purified perchlorate reductase from *Dechloromonas agitata*. It was hypothesized that the purified perchlorate reductase would stoichiometrically reduce any perchlorate in the sample to chlorite and the chlorite could be quantified by the chlorite specific assay producing a readily measurable yellow color.

In this format the development of the perchlorate assay was to be achieved under the following tasks. Two crucial go/no go decision points would be reached at the end of years 1 and 2 respectively, at which point a decision would be made to continue with the assay development.

Tasks

1. Purify the active perchlorate reductase (PR) from *D. agitata*.
2. Characterize the purified PR
3. Optimize and standardize the perchlorate assay protocol using the purified PR and the chlorite bioassay
4. Determine the lower detection limits of the assay
5. Determine the interference potential of soluble cations and anions
6. Test the robustness of the assay with environmental aqueous, soil, and sediment samples
7. Overexpress the PR in *E. coli* and characterize the recombinant protein

Background.

In recent years perchlorate has become a household word for the American public as concerns about its presence in water supplies throughout the US have resulted in communal outcry. These concerns have been further fueled by articles published in the popular press (Waldman, 2003) recounting disputes between the US EPA and the Pentagon regarding the reporting and regulation of this contaminant (Hogue, 2003; Renner, 2003). Furthermore, the findings of recent studies have indicated that the true extent of perchlorate contamination was severely

underestimated (Christen, 2003; Collette et al., 2003; Motzer, 2001; Orris et al., 2003) and the recent identification of its presence in major vegetable and dairy food products indicates that perchlorate may represent an even greater health threat than was previously considered (Kirk et al., 2003), (URL: http://www.ewg.org/reports/suspectsalads/).

Toxicity and regulation.

Perchlorate (ClO_4^-) is composed of a central chlorine atom surrounded by a tetrahedral array of four oxygen atoms. It is known to affect mammalian thyroid hormone production (Stanbury and Wyngaarden, 1952) and its toxicity predominantly results from its structural similarity to iodate (IO_4^-) which plays an important regulatory role in hormone production by the thyroid gland (Clark, 2000; Wolff, 1998). Thyroid hormone deficiency has a direct impact on neuropsychological fetal and infant development and studies have indicated that children of mothers suffering from maternal thyroid deficiency during pregnancy performed below average on 15 tests relating to intelligence, attention, language, reading ability, school performance, and visual-motor performance (Haddow et al., 1999). Prior to 1997, perchlorate was an unregulated compound in the US. However, as a result of the discovery of perchlorate contamination in drinking water resources throughout the US especially those in the southwestern states of Nevada, Utah, and California (Renner, 1998), the California Department of Health Services (DHS) together with the California EPA was prompted to establish a provisional action level of 18 $\mu g.L^{-1}$. In 1998 the US EPA added perchlorate to its Contaminant Candidate List for drinking water supplies (USEPA, 1998) and a final decision regarding the regulatory limit was to be set pending the outcome of ongoing toxicological studies (Renner, 1999). In January 2002, as a result of the publication of the first draft of the US EPA review on toxicological and risk assessment data associated with perchlorate contamination, a revised and lowered health protective standard of 1 $\mu g\ L^{-1}$ was suggested. Since the findings of this draft assessment were highly controversial to three other federal agencies, the US National Academy of Sciences (NAS) was asked to make an assessment. In January of 2005 the NAS suggested a maximum permissible dose of 0.7 $\mu g\ kg^{-1}\ d^{-1}$. This suggestion correlates to a standard of ~ 23 $\mu g\ L^{-1}$ for a normal adult person. However, the level would be lower for infants and children based on weight. This is especially poignant due to the recent study done on breast and dairy milk in the US. This study showed that perchlorate was detected in almost all milk samples analyzed. The

highest level detected in breast milk was 92 μg L^{-1}, a level 20 times higher than the NAS estimated maximum permissible dose for a baby. Reports of this magnitude are pressuring officials to set a final regulatory limit in the near future.

Sources of perchlorate

Perchlorate is principally a synthetic compound and its salts have a broad assortment of industrial applications ranging from pyrotechnics to lubricating oils (Motzer, 2001). Its presence in the environment predominantly results from legal historical discharge of unregulated manufacturing waste streams, disposal pond leaching, and from the periodic servicing of military inventories (Urbansky, 1998; Urbansky, 2002). To date, the only significant natural source of perchlorate characterized is associated with mineral deposits found in Chile where the perchlorate content averages as much as 0.03% of the total mineral mass (Ericksen, 1983). Throughout the last century, these Chilean ore deposits have been extensively mined as a mineral and nitrate source for fertilizer manufacture and the perchlorate often persists into the final product in low concentrations (Schilt, 1979; Urbansky et al., 2001). Although the full extent of the historical use of Chilean ore-based fertilizers is unknown in the US, currently their usage represents less than 0.2% of the USA fertilizer consumption (Collette et al., 2003) and recent modifications to the refinement process have significantly reduced the perchlorate content of these products (Urbansky et al., 2001). As such, these fertilizer products are not thought to represent a significant source of perchlorate in the environment (Urbansky et al., 2001).

In contrast, however, the presence of perchlorate in a variety of other natural potash-bearing evaporite samples collected from a diversity of arid locations has been indicated (Orris et al., 2003). More recently, it was demonstrated that solid fertilizers not derived from Chilean caliche and commonly used for the hydroponic growth of various fruit and vegetables may contain perchlorate at concentrations as high as 350 mg.kg^{-1} (Collette et al., 2003). Such levels could represent a significant global health threat due to the increasing use of hydroponic farming techniques for the production of a wide variety of plants for human consumption throughout the world (Collette et al., 2003). Studies performed on different plant species grown in soils containing perchlorate have indicated uptake (Ellington et al., 2001; Susarla et al., 2000; Van Aken and Schnoor, 2002) and in some cases transformation (reduction to chlorate (ClO_3^-),

chlorite (ClO_2^-), and chloride (Cl^-)) in the plant tissues (Susarla et al., 2000; Van Aken and Schnoor, 2002). In certain plant species such as tobacco and lettuce the perchlorate accumulated and persisted during processing into the final shelf products such as cigarettes, cigars, and chewing tobacco at concentrations as high as 60 mg.kg^{-1} (Ellington et al., 2001).

As these sorts of studies continue it is anticipated that other natural sources of perchlorate will be identified. For example, recent reports have indicated low-level perchlorate in drinking water wells of the southern part of Texas that exceeds a 30,000 square mile area (Rao et al., 2007). This perchlorate is known not to be associated with industrial activities or agricultural fertilizer application suggesting that this may also originate from an unidentified natural geological source (Rao et al., 2007). However, anthropogenic sources such as exists in Henderson, Nevada and the lower Colorado River (Hogue, 2003) will probably remain the principal culprit for the presence of perchlorate in water supplies. As of April 2003, perchlorate was manufactured and used in more than 150 industrial facilities throughout the US and more than 90 perchlorate releases have been reported in twenty five states. Ammonium perchlorate represents approximately 90% of all perchlorate salts manufactured (Motzer, 2001). It is predominantly used by the munitions industry and the US Defense Department as an energetics booster or oxidant in solid rocket fuels (Motzer, 2001; Roote, 2001; Urbanski, 1988; Urbansky, 1998). Although a powerful oxidant, under most environmental conditions perchlorate is highly stable and non-reactive due to the high energy of activation associated with its reduction (Urbansky, 1998; Urbansky, 2002). Because of the large molecular volume and single anionic charge, perchlorate also has a low affinity for cations and as a result, perchlorate salts, such as ammonium perchlorate, are generally highly soluble and completely dissociate into NH_4^+ and ClO_4^- in aqueous solutions. Furthermore, perchlorate does not sorb to any significant extent to soils or sediments and in the absence of any biological interactions its mobility and fate are largely influenced by the hydrology of the environment (Urbansky and Brown, 2003). Because of its unique chemical stability and solubility under environmental conditions microbial reduction has been identified as the most feasible means of remediating perchlorate contaminated sites (Urbansky, 1998).
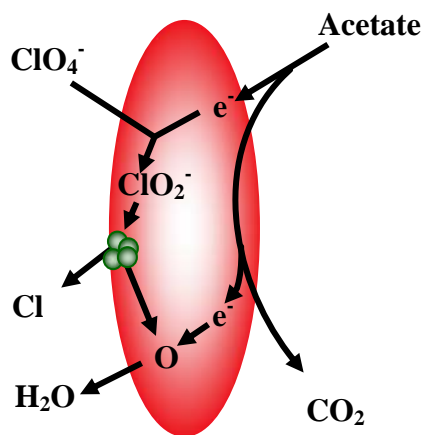
Microbial perchlorate reduction.

It has been known for more than fifty years that microorganisms can reduce oxyanions of chlorine such as chlorate (ClO_3^-) and perchlorate (ClO_4^-) [(per)chlorate] under anaerobic conditions (Aslander, 1928). The high reduction potential of (per)chlorate makes them ideal electron acceptors for microbial metabolism (Coates et al., 2000). Early studies indicated that unknown soil microorganisms rapidly reduced chlorate that was applied as an herbicide for thistle control (Aslander, 1928) and the application of this reductive metabolism was later proposed for the measurement of sewage and wastewater biological oxygen demand (Bryan, 1964; Bryan, 1966; Bryan and Rohlich, 1954). Initially it was thought that chlorate reduction was mediated by nitrate-respiring organisms in the environment with chlorate uptake and reduction simply being a competitive reaction for the nitrate reductase system of these bacteria (de Groot and Stouthamer, 1969; Hackenthal, 1965; Hackenthal et al., 1964). This was supported by the fact that many nitrate-reducing organisms in pure culture were also capable of the reduction of perchlorate (de Groot and Stouthamer, 1969; Hackenthal et al., 1964; Roldan et al., 1994). Furthermore, early studies demonstrated that membrane-bound respiratory nitrate reductases and assimilatory nitrate reductases could alternative reduce chlorate and presumably perchlorate (Stewart, 1988), and selection for chlorate resistance has been used as a screening tool to obtain mutants that are unable to synthesize the molybdenum cofactor required for nitrate reduction for many years (Neidhardt et al., 1996). However, chlorite (ClO_2^-) was produced as a toxic end product and no evidence was provided that nitrate-reducing organisms could grow by this metabolism.

Now it is known that specialized organisms have evolved which can grow by the anaerobic reductive dissimilation of (per)chlorate into innocuous chloride. Many dissimilatory (per)chlorate-reducing bacteria (DPRB) are now in pure culture (Coates and Achenbach, 2004). These organisms have been isolated from a broad diversity of environments including both pristine and contaminated soils and sediments (Coates and Achenbach, 2004). Phenotypic characterization revealed that the known dissimilatory (per)chlorate-reducing bacteria exhibit a broad range of metabolic capabilities including the oxidation of hydrogen, simple organic acids and alcohols, aromatic hydrocarbons, hexoses, reduced humic substances, both soluble and insoluble ferrous iron, and hydrogen sulfide (Coates and Achenbach, 2004). All of the known

DPRB are facultatively anaerobic or microaerophilic which is reasonable in light of the fact that molecular oxygen is produced as a transient intermediate of the microbial reduction of perchlorate (Coates and Achenbach, 2004). Some, but not all, DPRB alternatively respire nitrate (Coates and Achenbach, 2004).

Perchlorate reducing bacteria isolated to date are phylogenetically diverse (Coates et al., 1999; Michaelidou et al., 2000; Wallace et al., 1996) with members in the alpha, beta, gamma, and epsilon subclasses of the Proteobacteria phylum (Coates and Achenbach, 2004). The DPRB of the beta subclass of the proteobacteria represent two novel genera with monophyletic origin, the *Dechloromonas* species and the *Azospira* (formally *Dechlorosoma*) species (Coates and Achenbach, 2004). The *Dechloromonas* genus can be further subdivided into the RCB-type and CKB-type based on signature nucleotide sequences within the 16S rRNA gene sequence. Members of both the *Dechloromonas* and *Azospira* genera are ubiquitous (Coates and Jackson, 2008) and have been identified and isolated from nearly all environments screened including pristine and contaminated field samples, and even in soil and lake samples collected from Antarctica (Bender et al., 2004). As such, these two groups are considered to represent the dominant perchlorate-reducing bacteria in the environment (Coates and Jackson, 2008).

Although there is still relatively little known about the biochemistry of (per)chlorate reduction in general, some recent studies have yielded important information regarding the pathway and some of the central components involved. It is now accepted that perchlorate is initially reduced to chlorite which is further dismutated into chloride and oxygen (Coates et al., 1999; Rikken et al., 1996; van Ginkel et al., 1996). The oxygen is then respired to H_2O in a reductive metabolism (Achenbach et al., 2006; Coates et al., 1999; Rikken et al., 1996; van Ginkel et al., 1996) (Fig. 2). Initial investigations have demonstrated the presence of c-type cytochrome(s) in perchlorate-reducing bacteria and their involvement in the reduction of (per)chlorate (Bruce et al., 1999; Coates et al., 1999). Difference

Figure 2. The known steps of perchlorate reduction.

11

spectra studies (text box) revealed that the H_2-reduced c-type cytochrome content of different DPRB was readily reoxidized in the presence of chlorate or perchlorate but was unaffected by non-physiological electron acceptors for these organisms such as sulfate, fumarate, or Fe(III) suggesting their specific involvement in the transfer of electrons to (per)chlorate (Bruce et al., 1999; Coates et al., 1999).

More recently, an oxygen-sensitive perchlorate reductase from the (per)chlorate-reducer strain GR-1 has been purified and partially characterized (Kengen et al., 1999). This enzyme was located in the periplasm of the organism and was a trimer of heterodimers in an $\alpha_3\beta_3$ configuration composed of 95 kDa α subunits and 40 kDa β subunits (Kengen et al., 1999). The perchlorate reductase (PR) had a total molecular mass of 420 kDa and contained iron (30.6 mol), molybdenum (3 mol), and selenium (3 mol) per mole PR (Kengen et al., 1999). Although the presence of selenium is unusual for a reductase, several formate dehydrogenases are known to contain selenium (Heider and Bock, 1993). Subsequent phenotypic studies demonstrated that although selenium can be replaced with alternative cations for effective perchlorate reduction by DPRB (JD Coates, unpublished), the molybdenum plays a prerequisite functional role in the reduction of perchlorate (Chaudhuri et al., 2002). In addition to perchlorate, the perchlorate reductase from strain GR-1 also catalyzed the reduction of chlorate, nitrate, iodate, and bromate (Kengen et al., 1999). Perchlorate and chlorate were reduced to chlorite although the reductase activity was threefold higher toward chlorate than perchlorate (Kengen et al., 1999). Perchlorate was reduced to chlorite with a K_m value for perchlorate of 27 ± 7 µM and a V_{max} of 3.8 ± 0.4 Umg^{-1}, indicating that the purified enzyme is very efficient at reducing perchlorate (Kengen et al., 1999).

In contrast to the PR, the chlorate specific reductase recently purified from the chlorate-reducing bacterium *Ideonella dechloratans* exhibited no activity toward perchlorate (Danielsson-Thorell et al., 2003). The native 160 kDa enzyme was comprised of an α subunit (94 kDa), β subunit (35.5 kDa) and γ subunit (27 kDa) in an $\alpha\beta\gamma$ configuration. Similarly to the perchlorate reductase, the chlorate reductase (CR) also contained iron and molybdenum and, in addition to chlorate, also catalyzed the reduction of bromate, iodate, nitrate and selenate (Danielsson-Thorell et al., 2003). Chlorate was reduced to chlorite. Interestingly, both the CR and the PR reduced

chlorate and bromate at comparable rates respectively. Despite these similarities, these enzymes and the genes encoding them were distinct and only shared as much sequence similarity with each other as with other members of the DMSO reductase family. Although both chlorate-reducing and perchlorate-reducing bacteria produce a highly conserved chlorite dismutase to complete the respective reductive pathways, no organism to date has been identified that produces both a chlorate reductase and perchlorate reductase.

The quantitative dismutation of chlorite into chloride and O_2 is now known to be a central step in the reductive pathway of perchlorate that is common to all DPRB (Fig. 2) (Achenbach et al., 2006). Chlorite dismutation by DPRB is mediated by a highly-conserved single enzyme, chlorite dismutase (Achenbach et al., 2006). Studies with washed whole cell suspensions demonstrated that the CD was highly specific for chlorite and none of a broad range of alternative analogous anions tested served as substrates for dismutation (Bruce et al., 1999). The purified CD from *Dechloromonas* strain CKB was a homotetramer with a molecular mass of 120 kDa and a specific activity of 1,928 μmol chlorite dismutated per mg of protein per minute (Coates et al., 1999). This is similar to the molecular mass and specific activity observed for the CD previously purified from the DPRB strain GR-1 (van Ginkel et al., 1996) and subsequently from *Ideonella dechloratans* (Stenklo et al., 2001).

Perchlorate analysis
Remediation efforts of perchlorate contamination have focused primarily on microbial processes because of its unique chemical stability and high solubility (Urbansky, 1998). In 2001, a report published by Ground Water Remediation Technologies Analysis Center (Roote, 2001) outlined 65 different case studies of perchlorate treatment technologies for targeting contaminated wastewater, surface water, groundwater, and soils. The majority (45 case studies) were either in-situ or ex-situ biological treatment technologies based on the unique ability of some microorganisms to reductively respire perchlorate completely to innocuous chloride in the absence of oxygen (Roote, 2001). Other physical/chemical technologies such as adsorption by activated charcoal, reverse osmosis, or ion exchange have proved difficult or failed because of rapid saturation of active sites or the high cost, especially that associated with the processing of surface or groundwater contamination where excessively large volumes containing low levels of

perchlorate may require treatment. In addition, physical removal processes such anion exchange still require subsequent disposal of removed perchlorate.

The optimum treatment technology for a given perchlorate occurrence may depend on several factors, including perchlorate concentration, the presence and concentration of co-contaminants, other water quality parameters (pH, alkalinity, natural organic matter (NOM), total dissolved solids (TDS), metals, etc.), and geochemical parameters (nitrate, sulfate, chloride, dissolved oxygen, redox potential, etc.). The presence of indigenous dissimilatory perchlorate-reducing bacteria (DPRB), and substances inhibitory to DPRB activity will also influence biological perchlorate treatment technology effectiveness. For in situ treatment of perchlorate contamination, variables related to the site hydrogeological setting, such as depth and distribution of contaminants, soil permeability, groundwater flow velocity, etc. are also additionally important.

The successful implementation of any remediative strategy is dependent on the accurate identification of the boundaries of the perchlorate plume. The most common method currently available is a sensitive ionic chromatographic analytical technique with conductivity detection that was developed in the mid 1990's (Wirt et al., 1998), and which forms the basis of the current EPA Method 314 for the determination of perchlorate in drinking water (Hauntman et al., 1999). Although accurate for elevated concentrations, the lower limit of detection for this method is approximately 4 μg.L^{-1} which is four times the recommended MCL limit set by the EPA in 2002. Furthermore, perchlorate identification is based on chromatography elution times in comparison with standards rather than specific molecular structure which leaves a significant margin for interference and error.

More recently several alternative more sensitive and accurate techniques including complexation electrospray mass spectrometry, tandem electrospray mass spectrometry, high-field asymmetric waveform ion mobility spectrometry, and Raman spectrometry have been developed and applied to a broad range of environmental samples (Collette et al., 2003; Urbansky, 1998; Urbansky, 2002; Urbansky and Brown, 2003), and references therein. These techniques have proved to be

exceptionally accurate for identification of perchlorate concentrations in the sub-ppb range in many complex matrixes (Collette et al., 2003; Urbansky and Brown, 2003).

However, all of the techniques currently available are laborious, expensive, time consuming, and require highly trained personnel making them unsuitable for the rapid delineation of contaminated environments. In our original proposal we proposed to develop an alternative biochemical technique based on combining the purified perchlorate reductse from the perchlorate reducer *Dechloromonas agitata* strain CKB with a enzymatic colorimetric assay previously developed by the PI's lab for the sensitive determination of chlorite (Fig. 3) (O'Connor and Coates, 2010). It was proposed that the perchlorate reductase would quantitatively reduce any perchlorate present to chlorite, which could be detected by the enzymatic chlorite bioassay. This was hypothesized to provide a rapid, specific, and sensitive method for the detection of perchlorate that would obviate the need for expensive equipment (e.g. ion chromatograph).
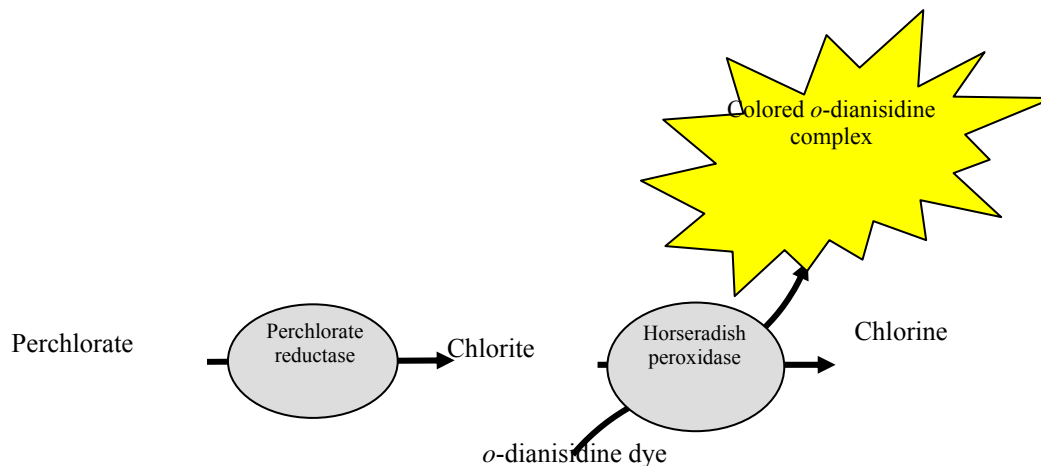
Figure 3. Model of the biochemistry of the proposed perchlorate bioassay

The chlorite bioassay

Previously, as part of ongoing research in the PI's lab into the microbial interactions with oxyanions of chlorine, a simple colorimetric assay for the determination of low-level concentrations of chlorite was developed. The assay was based upon the enzymatic reaction between chlorite and horse radish peroxidase (HRP) which converted the chlorite quantitatively

into chlorine dioxide. The chlorine dioxide formed can then be determined colorimetrically upon reaction with a dye such as o-dianisdine or lissamine green to form a colored complex. The color formed was directly proportional to the initial chlorite concentration. In the case of the o-dianisidine a yellow product was produced which can be determined spectrophotometrically at 450nm. This assay has already been optimized for rapid robust application and because of its ease of use, extreme sensitivity, and low cost the US EPA has adopted an adapted version (method 327) as the standard method for the monitoring of chlorite in drinking water samples. Our previous studies revealed that the optimum pH for the assay was pH 7.3 as it gave the most linear curve over the concentration range tested. The assay was also optimized for incubation temperature (25 ^{o}C) and time (25 min). The assay is sensitive to chlorite concentrations as low as 0.4 micromolar (approx 27 ppb) and is linear and reproducible up to 5.0 millimolar. Screening tests revealed that the assay did not suffer from interference by the presence of common ions such as nitrate, nitrite, sulfate, ferric iron, bromate, or chlorine. It can be applied in one of two forms for the detection of chlorite, either as a quantitative colorimetric assay or as a semi-quantitative dipstick. As such the combination of this assay with the purified perchlorate reductase and a suitable electron donor (e.g. NADH) would allow the simple, rapid analysis of perchlorate present in environmental samples. Assuming that the purified perchlorate reductase enzyme quantitatively converts perchlorate to chlorite, the combined assay was theorized to immediately give a linear sensitivity for perchlorate concentrations as low as 40 ppb without interference. Although this lower measurable concentration of perchlorate is above the targeted 1 μg.L^{-1} maximum concentration level, a basal amount of perchlorate (35 ppb) can be added into the assay system to ensure that the bioassay can measure perchlorate concentrations down to the current MCL goal.

Initial conceptual flaw and correction
Our original project concept was based on recent advances in our understanding of the biochemistry and genetics of microbial perchlorate reduction. Our goal was to take advantage of the activity of the primary enzyme involved in the biochemical pathway of perchlorate reduction, the perchlorate reductase, which quantitatively reduces perchlorate to chlorite. We had proposed to couple the activity of the perchlorate reductase enzyme to a bioassay we had previously developed for chlorite. The purified perchlorate reductase stoichiometrically reduces any

perchlorate in the sample to chlorite and the chlorite can then be quantified by the previously described chlorite assay producing a readily measurable yellow color (Fig. 3). Unfortunately, due to an unpredicted chemical reaction between the electron donor of the perchlorate reductase and the chlorite formed by the enzyme, this approach was unlikely to prove successful (Fig. 4).
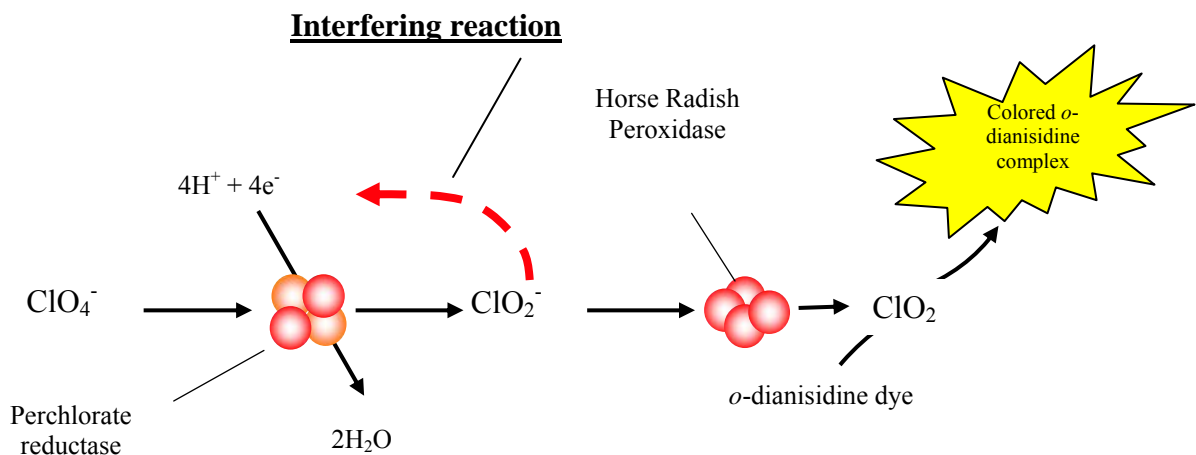
Figure 4. Model of the biochemistry of the originally proposed bioassay showing the unforeseen interfering abiotic reaction between chlorite and the residual perchlorate reductase electron donor

However, the reactivity of the chlorite with the primary electron donor may yield an unforeseen advantage. If transformation of the electron donor (e.g. dithionite; $S_2O_4^{2-}$) is monitored then the abiotic reaction between the chlorite formed and the residual electron donor available (reaction 2 below) would result in an inherent signal amplification in the assay which should significantly enhance the assay sensitivity for perchlorate (reactions 1 and 2 below combined).

$ClO_4^- + 2S_2O_4^{2-}$ (electron donor) $+2H_2O \xrightarrow{\text{Perchlorate reductase}} ClO_2^- + 4SO_3^{2-} + 4H^+$ …….. Rxn 1

$ClO_2^- + 2S_2O_4^{2-}$ (electron donor) $+ 2H_2O \xrightarrow{\text{Abiotic reaction}} Cl^- + 4SO_3^{2-} + 4H^+$ …………..Rxn 2
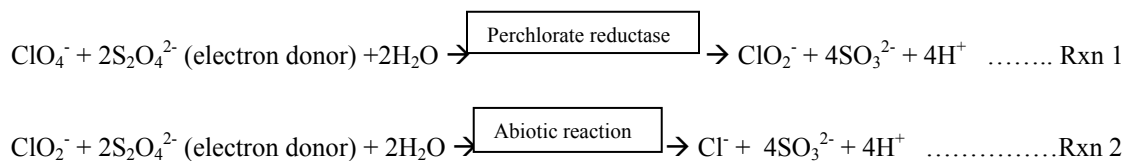
This new conceptual direction of the project did not alter the original tasks performed. The major changes made were to task 3, which was designed to optimize and standardize the perchlorate bioassay protocol. Under the revised conceptual plan the perchlorate bioassay was based on a three-step process (i) extraction of the perchlorate; (ii) reaction of the perchlorate with

the perchlorate reductase using a suitable electron donor; (iii) quantification of the electron donor consumed in step ii by the perchlorate reductase.

Materials and Methods.

Overview.

A highly sensitive perchlorate reductase enzyme was purified by our laboratory from *Dechloromonas agitata* strain CKB previously grown under perchlorate reducing conditions (20 mM acetate, 20mM perchlorate). The oxygen labile enzyme was purified from the soluble cell fraction under an anoxic atmosphere (100% N_2). Purified perchlorate reductase had a specific activity of 1.10 ± 0.093 U/mg (ClO_4^-) and 3.35 ± 0.10 U/mg (ClO_3^-). Consistent with previous reports, purification resulted in the loss of the c-type cyctochrome subunit, pcrC, as revealed by oxidized minus reduced difference spectra of the purified protein.
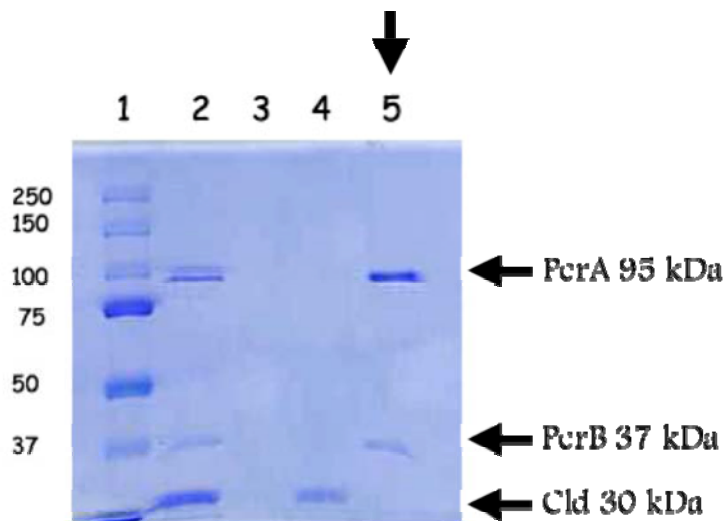
Cell culturing.

A large scale culturing technique was developed which allowed us to regularly culture 200L of *Dechloromonas agitata* strain CKB anaerobically. These cultures were grown at 25^oC with 20mM acetate as the electron donor and 20mM perchlorate as the primary electron acceptor. The active cells were harvested at late log phase by continuous centrifugation yielding 240 g wet weight cell paste. The active cell paste was sub-aliquoted into approximately 15 g quantities and stored by blast freezing in liquid N_2.

Cell lysate preparation.

All procedures were performed under a constant stream of N_2 to reduce oxygen exposure of the oxygen labile enzyme. 15g of CKB cells were thawed out at room temperature and resuspended 50mM phosphate buffer (15 mL). Cells were then lysed by passage through a French pressure cell at 16,000 psi. The cell extract was pelleted by ultracentrifugation at 15k RPM. The collected pellet was washed by resuspenion in 50mM phosphate buffer and repelleted. The collected supernatant fractions were combined and subjected to ultracentrifugation at 45k RPM for 1hr. Anaerobic glycerol was added to the red supernatant (soluble fraction) to give a final concentration of 10% by volume.

Protein purification. The perchlorate reductase was purified from the prepared cellular soluble fraction by sequential column chromatography in chilled glass columns under a constant stream of N_2. The active fraction containing perchlorate reductase was identified by a methyl viologen (MV) based assay in which the oxidation of reduced (blue) MV to oxidized MV (colorless) was monitored spectrophotometrically at 578 nm in the presence of perchlorate. The initial soluble fraction from the cell lysate was loaded onto an SP-Sepharose column

Figure 5. 10% SDS-PAGE gel of the hydroxyappetite step. Lane 1, protein markers with molecular weights given; lane 2, SP Sepharose pool loaded onto the column; lane 3, non-bound pool; lane 4, chlorite dismutase eluted at the beginning of the potassium phosphate gradient; lane 5, two subunits of the perchlorate reductase eluted at the end of the gradient. Proteins in the gel were visualized with Coomassie brilliant blue R250.

(3.2 x 13cm, 100mL) previously equilibrated in anaerobic 50mM phosphate buffer pH 7.2 containing 10% glycerol. Perchlorate reductase co-eluted with the chlorite dismutase (30% of the total protein mass of the cell) in the middle of a linear gradient (300mL) of 0 to 1 M potassium chloride in 50mM phosphate. The fractions containing perchlorate reductase were pooled and subsequently passed through a column of hydroxyappetite (1x6cm, 5mL Bio-Scale CHT5-I). In this instance the perchlorate reductase eluted from the column at the end of a linear gradient (100mL) of 10 to 450 mM potassium phosphate, separated now from the majority of the chlorite dismustase. The active fractions were again pooled and finally loaded (in 2 ml aliquots) onto a Superdex200 column (1.6 by 70.5) equilibrated in 50mM MOPS buffer, pH 7.0 containing 10% glycerol and 100mM KCl. The purified perchlorate reductase eluted from the column in a single symmetrical brown peak. Protein purification was confirmed by SDS-PAGE gel electrophoresis at each stage of the chromatography (Fig. 5). The perchlorate reductase, a known heterotrimeric protein, yielded only two bands at 95 and 37 kDA, representing the PcrA and PcrB subunits, respectively, indicating that the loosely associated PcrC heme protein of the original enzyme was lost during the purification protocol.

Protein analyses.

The amino terminus of PcrA has a twin-arginine motif targeting it for secretion to the periplasm (5b). The SignalP program predicts a signal peptide of 28 amino acids, leading to a start sequence of ATMDL. Based on the translated gene sequences, the predicted PcrABC subunit molecular weights are respectively 102, 37, 25 kDa, leading to a predicted molecular weight of ~165 kDa for the 3-subunit complex. Isoelectric focusing determined an approximate isoelectric point of 9.6 for Pcr, relative to standards, indicating that the protein bears a net positive charge at neutral pH (Fig. 6).

Chemical analyses.

Purified enzyme was analyzed for metal, sulfide and cofactor (molybdopterin and heme) content in collaboration with Prof. J. Dubois of Notre Dame University, IN. Assuming a PcrABC subunit stoichiometry of 1:1:1 (total MW of 165 kDa), the protein was found to contain 22.7 ± 0.40 Fe, 1.19 Mo ± 0.34, and 21.7 ± 2.5 S^{2-} per molecule (note: errors reported are standard deviations for triplicate [Mo, Fe] or 6-sample [S] measurements). However, SDS-PAGE of the purified protein indicates that PcrC is partially or completely lost during purification. If it is

Figure 6. Isoelectric focusing gel of purified Pcr. Standards run in outermost lanes and four lanes of Pcr in the center lanes. Standards are (from top): cytochrome c (pI = 9.6), lentil lectin (3 bands: pI = 8.2, 8.0, 7.8), human hemoglobin C (pI = 7.5); human hemoglobin A (pI = 7.1); equine myoglobin (2 bands: pI = 7.0, 6.8); human carbonic anhydrase (pI = 6.5); bovine carbonic anhydrase (pI = 6.0); β-lactoglobulin B (pI = 5.1); phycocyanin (3 bands: pI = 4.75, 4.65, 4.45).

assumed that the pure protein is primarily the dimeric PcrAB complex with an approximate molecular weight of 140 KDa, the metal:protein stoichiometries are then: 19.3 ± 0.34 Fe, 1.01 ± 0.29 Mo, and 18.4 ± 2.1 S^{2-}. These numbers are within error or close to the per-PcrAB values of 15 equivalents Fe, 16 equivalents sulfide, and 1 equivalent of molybdenum predicted from sequence analysis. The absence of PcrC is also consistent with the failure to detect heme, even at expected PcrC concentrations of 2.2 mM, via the highly sensitive alkaline pyridine hemochrome assay (measured $\varepsilon_{R-O} = 15.3$ mM^{-1}cm^{-1} at $\lambda = 555$ nm for the heme b standard).

SDS-PAGE analysis of protein fractions from the purification process indicate that, while a band corresponding to the molecular weight of PcrC is present following ion exchange, this band disappears after hydroxyapatite chromatography. The phosphate functionalities of the hydroxyapatite medium likely mimic the phospholipid bilayer, near which PcrC is proposed to reside. The high isoelectric point of the protein further suggests that strong affinity of the protein for hydroxyapatite is very likely. It is possible that strong interaction with hydroxyapatite PcrC is responsible for removal of the subunit from the protein.

Protein activity.
The specific activity of the purified enzyme was measured using ClO_4^- and ClO_3^- as substrates. Importantly, dithionite was added sub-stoichiometical*y* to reduce methyl viologen in situ. Sub-stoichiometric addition eliminated the possibility of nonenzymatic redox cycling between the reduced and oxidized forms of methyl viologen. Initial rates measured in this way for pure perchlorate reductase were: 1.10 ± 0.093 U/mg (ClO_4^-) and 3.35 ± 0.10 U/mg (ClO_3^-).

Results and Discussion

Initial Assay Development.
Two publications have used an assay to quantitate perchlorate activity anaerobically (Kengen et al., 1999; Okeke et al., 2002). In this assay the dye methyl viologen is reduced by sodium dithionite. Following this reduction, the dye is oxidized by perchlorate reductase in the presence of perchlorate. Although no evidence exists that perchlorate is truly reduced in this assay, it is assumed to occur because of the oxidation of methyl viologen. The second assumption that is made is that eight electrons are transferred from the reduced dye to perchlorate to form chloride.

Taking these assumptions to be true, preliminary experiments on perchlorate reductase were conducted. As shown in figure 7, the rate of methyl viologen oxidation per mg protein increases as perchlorate concentration increases. This increase appears linear between 0 and 50 µM perchlorate, but as the perchlorate concentration approaches 400 µM the rate ceases to increase linearly with increasing perchlorate concentrations.

When this data is analyzed using a lineweaver-burke plot, or double inverse plot, the correlation between the inverse specific activity and the inverse concentration of perchlorate is linear (Fig. 7). The linear relationship implies that the correlation between specific activity and perchlorate concentration follow Michaelis-Menten kinetics. This well characterized kinetic model can be
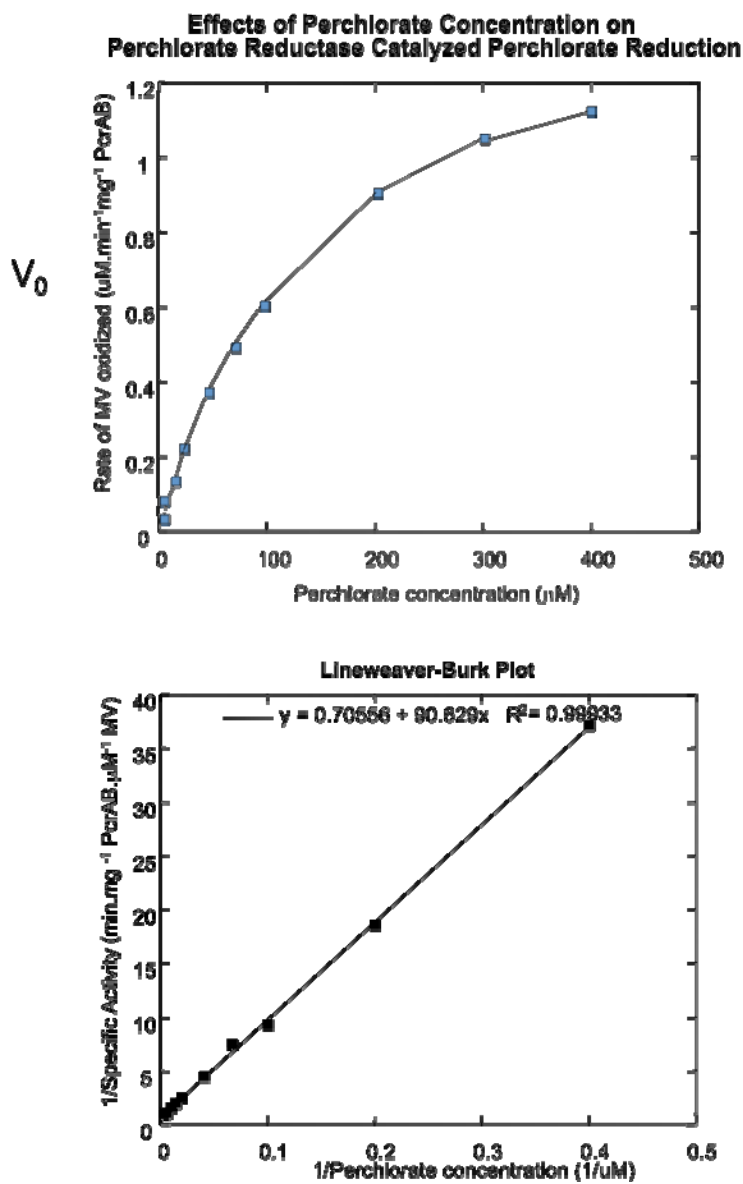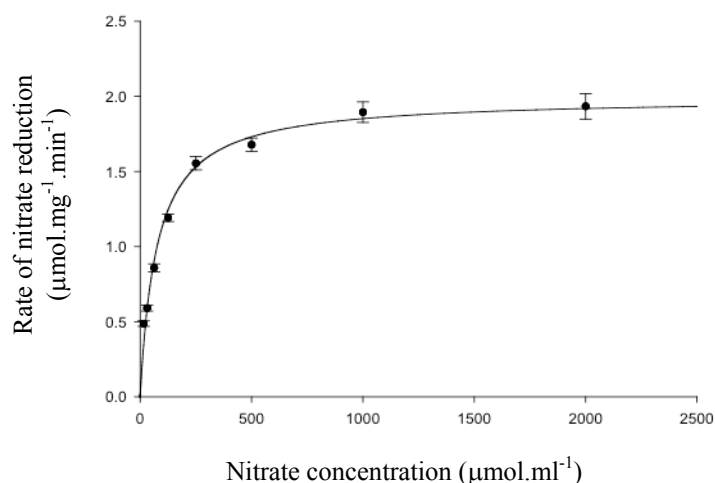

Figure 7. Effects of perchlorate concentration on perchlorate reductase catalyzed perchlorate reduction

used to quantitate perchlorate concentration, and demonstrates the feasibility of using this enzyme as part of a bioassay. Using this relationship, the important constants of K_M and V_{MAX} were determined to be 127 µM and 0.44 U, respectively.

Testing the Assay under Various Conditions.

The preliminary bioassay was tested in the presence of different contaminating ions, acidic and basic pH, salinities, and oxygen exposure in our anaerobic chamber. The point of these experiments was to determine the resiliency of the assay to disruptive sample conditions. In the first experiment, perchlorate reductase was incubated with nitrate and chlorate. These two ions are similar in structure to perchlorate, and previous studies have shown that in the presence of these ions perchlorate reductase can oxidize methyl viologen (Kengen et al., 1999). In addition, these two ions are commonly found in groundwater. Nitrate is a

Figure 8. Reaction of perchlorate reductase with nitrate . The V_{max} and K_m were determined to be 2U and 75 μM respectively.
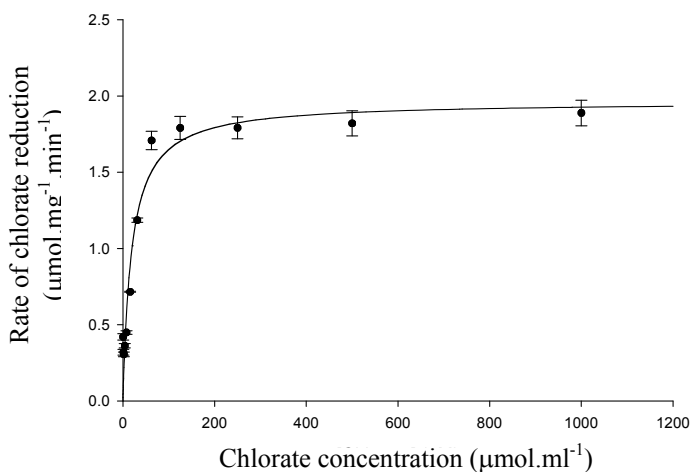
Figure 9. Reaction of perchlorate reductase with chlorate. The V_{max} and K_m were determined to be 2U and 19.1 μM respectively.

ubiquitous soil ion, and chlorate has been used in agriculture as an herbicide. As shown, in the presence of both nitrate (Fig. 8) and chlorate (Fig. 9) the enzyme oxidizes methyl viologen, and the relationship of the rate of this oxidation to ion concentration appears similar to or greater than the relationship found for the perchlorate ion. Although chlorate and nitrate have not been proven to be reduced by the enzyme, it can be inferred that this is the ultimate destination of the

23

electrons extracted from methyl viologen. These results indicated that any assay that attempts to use PCR to determine perchlorate concentration will have to have a purification step to remove the nitrate and chlorate.

In addition to contaminating ions, the assay sensitivity to changes in pH was determined. Changes in pH can have a devastating effect on enzyme activity by changing the conformation of the enzyme (Mathews et al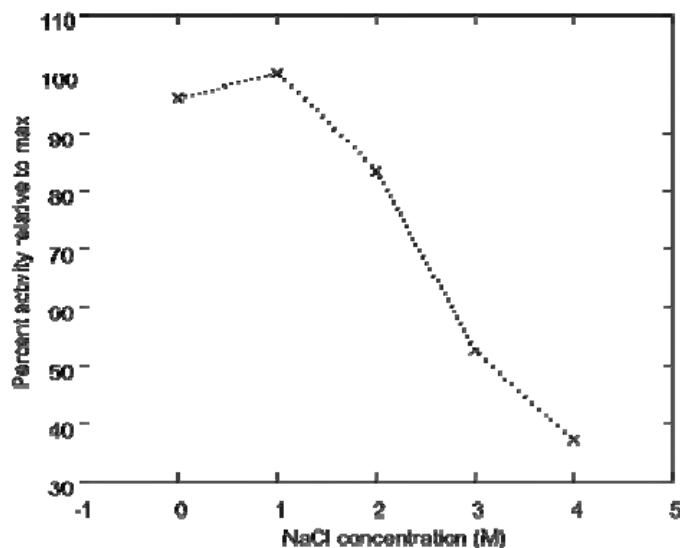., 1999). In addition, changes in pH can increase the redox potential of sodium dithionite, making the chemical less likely to reduce methyl viologen for the assay (Heinnickel et al., 2005). As can be seen in figure 10, the rate of methyl viologen oxidation is

Figure 10. pH profile for PcrAB activity. Data points were adjusted by subtracting the average of the negative controls for that pH from each data point**.**

maximized at pH 7-7.5. Solutions that are more acidic or basic than pH 7 have lower rates of methyl viologen oxidation in the presence of perchlorate. Although the enzyme is still active, it is clear that for maximal activity any sample would have to have its pH adjusted to ~7. A deviation of one pH unit from this optimal condition can decrease the rate of the reaction by 30-40%.

Another condition that commonly affects protein folding and activity is salinity (Mathews et al., 1999). The bioassay's resistance to salinity is very important, as ocean and seawater samples would contain high concentrations of salt (Stumm and

Figure 11. NaCl profile for PcrAB activity.

24

Morgan, 1996). As seen in figure 11, the enzyme activity did not change in the presence of 1 M NaCl. In the presence of 2 M NaCl the salinity has only a modest effect on the enzyme, decreasing it activity ~15%. Higher salinity did have an effect on the enzyme with 4 M NaCl decreasing the activity by 60%. However, the enzyme was still active demonstrating its robustness in high concentrations of NaCl. This attribute could be used in the development of an aerobic assay as NaCl decreases the solubility of oxygen in water.

Figure 12. Effect of atmospheric oxygen on perchlorate reductase activity over time

For this assay to be field ready it needs to be oxygen tolerant. It has been published previously that the enzyme is labile in the presence of oxygen (Kengen et al., 1999). In order to determine the enzyme's oxygen stability the enzyme was allowed to remain on the bench at room temperature for various amounts of time. As can be seen in figure 12, oxygen does have an effect on the enzyme. After a period of only 30 min, the enzyme's activity does not appear to be greatly affected (loss of ~5%). However, after a period of 2 hours the
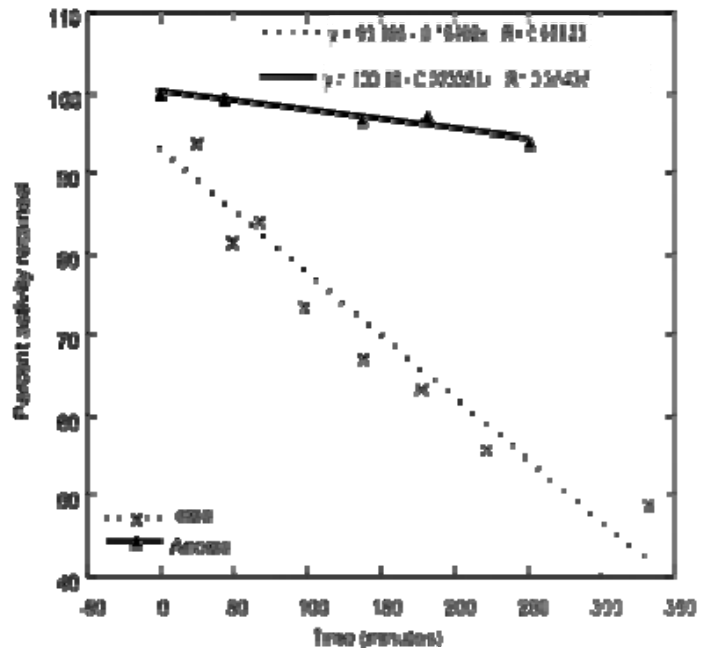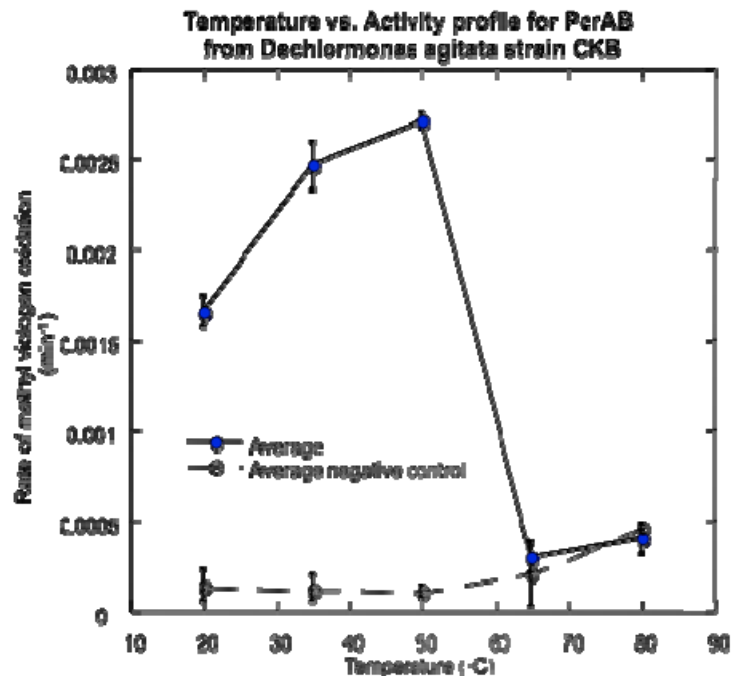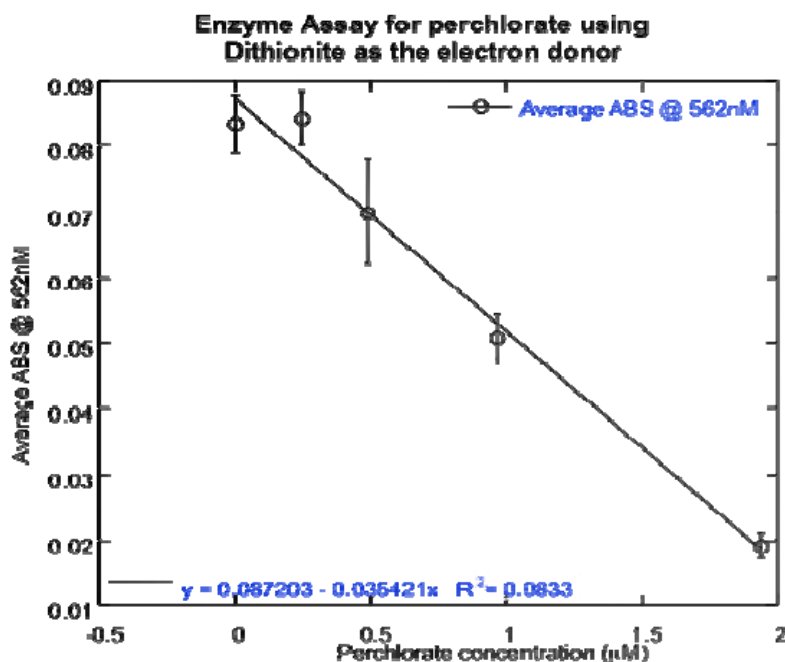

Figure 13. Effect of temperature on perchlorate reductase activity

enzyme's activity decreases much more significantly (loss of ~30%). Therefore, it seems clear that any assay that is developed will require an oxygen reduction step. This oxygen reduction step is important not only for the enzyme's stability, but for other components in the assay that may be labile in the presence of oxygen (dithionite and methyl viologen).

To further optimize the bioassay, experiments were carried out to elucidate the optimal temperature for activity. Although all previous experiments were performed at room temperature, when the assay is carried out at higher temperature, the enzyme's activity increases. As can be seen in figure 13, as temperature is increased to 50° C the enzyme's activity increases ~1.7 fold. However at ~60° C the enzyme's activity decreases by 75%, compared to the activity at room temperature. This decrease in activity indicates the enzyme has most likely been heat denatured at this temperature. However, it is interesting to note that by increasing the temperature it is possible to decrease the time required to detect perchlorate. As temperature increases, the rate of diffusion is also increased, and thus the rate of enzyme and substrate collision. This increase in rate will result in a lower binding constant, and therefore its decrease in assay detection limit.

Lower Detection Limits of an Enzyme Based Assay

The lowest concentration of perchlorate that was tested to react with the enzyme in the preliminary assay was 2.5 μM (250 ppb). This concentration is approximately ~40 times larger than the recommended drinking limit for California (6 ppb) (Fan et al., 2004). To determine if the enzyme could detect perchlorate at a level of 6 ppb, the bioassay protocol was modified to decrease our detection limit. In this

Enzyme Assay for perchlorate using Dithionite as the electron donor

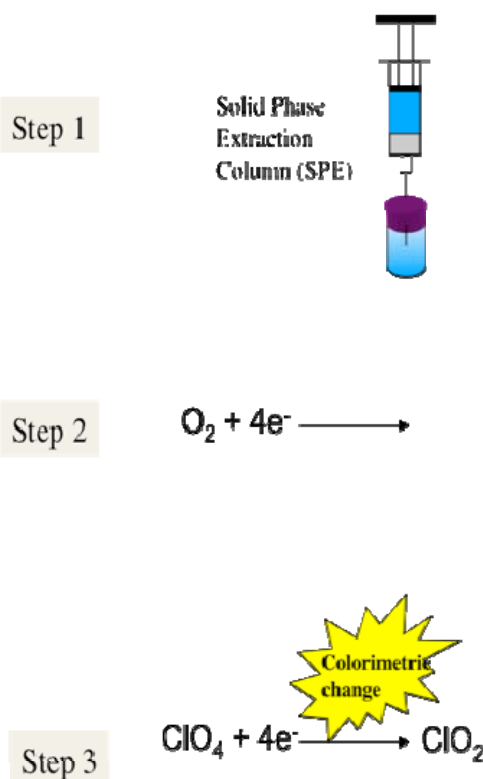$y = 0.087203 - 0.035421x \quad R^2 = 0.9833$

Figure 14. Use of the ferrozine assay to determine perchlorate concentration in the ppb range through a back titration mechanism with Fe(II) and dithionite. Although the assay would be difficult to apply for aerobic use, it proves the enzyme can be used to detect perchlorate in the ppb range.

alternative protocol, 9,10–Anthraquinone-2,6-Disulfonate (AQDS) replaced methyl viologen as electron shuttle. As the reaction proceeds, dithionite is oxidized by AQDS to produce the reduced hydroquinone form 9,10–anthrahydroquinone-2,6-disulfonate (AHDS). The AHDS thus formed subsequently is reoxidized to AQDS and transfers the electrons to PCR to reduce perchlorate. After a brief incubation (~20 min), the remaining dithionite can be quantified by reaction with Fe(III) to produce Fe(II) which is subsequently measured using a standardized ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid) assay. As ferrozine reacts with Fe(II) it produces an absorbance at 562 nm. Because of the complexity and oxygen sensitivity of ferrozine, this version of the bioassay must be done in an anaerobic chamber. For this reason, this assay is not suitable as a field assay to determine perchlorate concentrations. However as can be seen in figure 14, the obtained results demonstrated a reproducible absorbance change at 562 nm correlating to concentrations of perchlorate between 0.25 and 2 μM (25 – 200 ppb).

Strategy for a Field Assay

From the observations above, it was clear that any assay that uses PCR to detect perchlorate at concentrations similar to the recommended drinking limit will have to meet specific criteria: (1) The solution will have to be free of interfering ions such as nitrate and chlorate (2) Perchlorate in the solution will have to be concentrated 4 – 100 fold (depending on the assay) (3) The assay will have to have an oxygen removal step.

To overcome these difficulties we have assembled a general strategy that concentrates the perchlorate and removes all molecules that can contaminate the sample such as nitrate, chlorate, and molecular

Step 1 — Solid Phase Extraction Column (SPE)

Step 2 — $O_2 + 4e^-\longrightarrow$

Step 3 — $ClO_4 + 4e^-\longrightarrow ClO_2$ (Colorimetric change)

Figure 15. The general strategy for a perchlorate assay in the field. Step 1 is derived for a published protocol, data on steps 2 and 3 are detailed in the latter half of this report.

oxygen (Fig. 15). In the first step of this strategy, the sample is concentrated on a solid phase extraction column (SPE). A protocol to use SPE columns has already been published (Thorne, 2004). In brief, the sample is loaded onto a styrene-divinylbenzene (SDVB) cartridge conditioned with decyltrimethylammonium bromide (DTAB). The ions chlorate, nitrate, and perchlorate all bind to the resin. Chlorate and nitrate are eluted using 15% acetone and methanol, while perchlorate is eluted using 100% acetone. In addition, the SPE column can concentrate samples up to 500 fold. Following this purification and concentration, the sample must be reduced to remove all of the oxygen. The final step in this protocol is to determine perchlorate concentrations using a colorimetric bioassay.

Development of a colorimetric bioassay using stable reductants
Although there seems to be a correlation between perchlorate concentration and the rate of methyl viologen oxidation, it may be difficult to base an assay on rate changes. Therefore, we have tried to design an assay that correlates absolute absorption changes (amount of methyl viologen oxidized) with perchlorate reduced. We have hypothesized that perchlorate reductase would oxidize 4 molecules of methyl viologen to produce chlorite. The resulting chlorite will oxidize four additional molecules of methyl viologen. This reaction would result in an eight-fold signal amplification in perchlorate detection. In order to test this hypothesis various concentrations of reduced viologen were incubated with perchlorate reductase, and ~100 μM perchlorate. As can be seen in figure 16, when perchlorate reductase oxidizes between 0 and
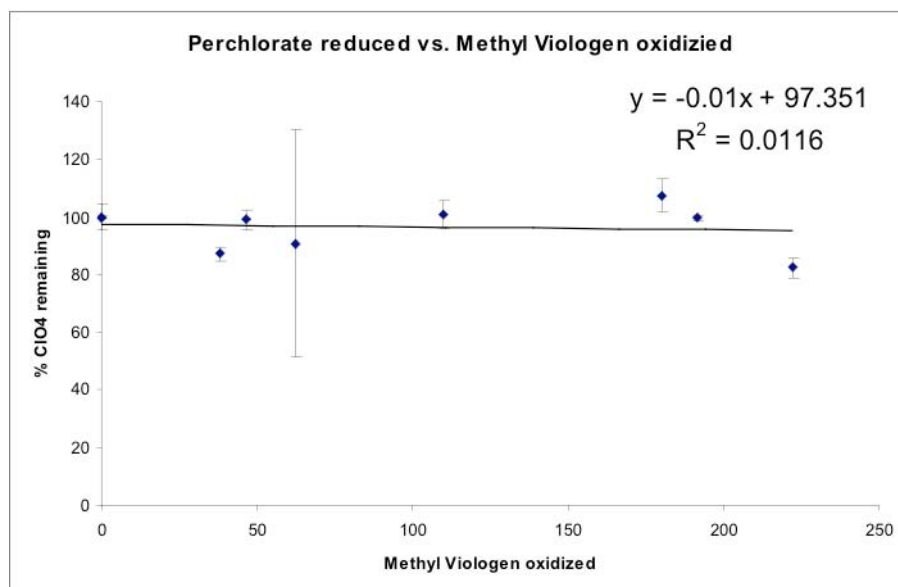
Figure 16. The perchlorate remaining following an incubation with perchlorate reductase and reduced methyl viologen.
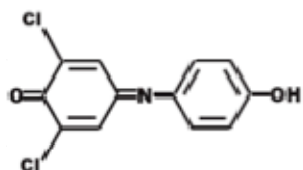
Dichlorophenol indophenol, $E^{0'}$ = +217 mV (DCPIP)
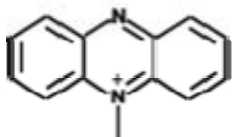
N-methyl Phenazine Methosulfate, $E^{0'}$ = +80 mV (PMS)
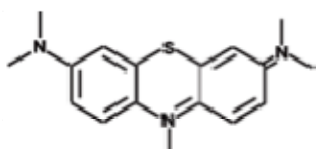
Methylene Blue, $E^{0'}$ = +11 mV (MB)
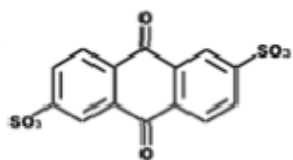
9,10-Anthraquinone-2,6-Disulfonate, $E^{0'}$ = -184 mV (AQDS)
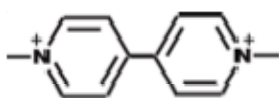
Methyl Viologen , $E^{0'}$ = -440 mV (MV)

Figure 17. Redox potential, structures, names, and abbreviations of dyes used in the dye screening experiment.

200 µM methyl viologen there is no discernable reduction of perchlorate (perchlorate concentrations were determined using an ICS 1500 ion chromatography system). When larger concentrations of reduced methyl viologen are used (0.8 or 1 mM) some reduction of perchlorate is observed, however, these amounts of reduced methyl viologen are outside the range of detection of any optical technique. The amount of methyl viologen oxidized in these large concentrations appears to be 12 molecules of methyl viologen per molecule of perchlorate reduced.

Although it is peculiar that the enzyme oxidizes methyl viologen in the presence of perchlorate, but does not reduce perchlorate, these results do not undermine the earlier findings. It seems clear that there is a correlation between the amount of perchlorate present and the rate of oxidation of methyl viologen. However, using this dye and reductant system to reliably quantify perchlorate concentrations will not be effective.

Other complications with this assay involve the use of sodium dithionite. Dithionite is a hygroscopic chemical, and when it is dissolved in water it is extremely oxygen labile. These characteristics complicate the use of

Figure 18. 50 µM of the indicated dyes were incubated with 122 µM perchlorate, 1 mM ascorbate and 45 µg.ml^{-1} PCR. The bar graph above shows the amount of perchlorate remaining after this incubation.

dithionite in any aerobic field assay. Therefore, it would be easier if this assay used a reductant that was not labile in the presence of molecular oxygen.

In an attempt to optimize and standardize a new perchlorate assay protocol, several alternative electron donors and dyes with a range of redox potentials were screened. As shown in figure 17,

experiments were conducted with dyes that span redox potentials of +217 mV to -440 mV (redox potentials determined from (Fultz and Durst, 1982)). The dye structures include an indole, a quinone, a bipyridinium, and two phenazine molecules (Fultz and Durst, 1982). All of these electron transfer agents are commonly used in biology, and especially photosynthesis (Fultz and Durst,

Perchlorate remaining after incubation with PCR and dithiothreitol

Figure 19. 50 μM of the indicated dyes were incubated with 122 μM perchlorate, 1 mM dithiotreitol and 45 μg.ml⁻¹ PCR. The bar graph above shows the amount of perchlorate remaining after this incubation.

1982). In figure 18, these dyes are incubated with 45 μg/ml PCR, 100 μM perchlorate, and 1 mM sodium ascorbate. Sodium ascorbate is a mildly oxygen labile dye that is inexpensive and has a redox potential of +58 mV (Mathews et al., 1999). The perchlorate concentration that remained after an incubation of one hour is shown on the y-axis in figure 18. The negative control (farthest to the left) has only perchlorate. The no dye control (second from the left) has enzyme and reductant, but no dye to shuttle electrons into the enzyme. The remaining four

samples have the components listed above, as well as 100 μM of the dye shown on the x-axis. As can be seen in figure 18, phenazine methosulfate (PMS, fourth from the left) is the most efficient dye as it reduces the most perchlorate in 1 hour. To a lesser extent, methylene blue (MB, second from the right) acts as a shuttle reducing ~50% of the perchlorate in 1 hour. It should be

Perchlorate remaining after incubation with PCR and dithionite

Figure 20. 50 μM of the indicated dyes were incubated with 122 μM perchlorate, 1 mM dithionite and 45 μg.ml⁻¹ PCR. The bar graph above shows the amount of perchlorate remaining after this incubation.

mentioned that PMS and MB have similar chemical structures fitting in the phenazine subclass of chemical dyes (Fultz and Durst, 1982). The dyes dichlorophenol indophenol (DCPIP, third from the left) and 9,10-anthraquinone-2,6-disulfonate (AQDS, first from the right) were largely ineffective at transferring electrons to PCR from ascorbate.

In figure 19 a similar dye experiment was conducted, but dithiothreitol was substituted as reductant for sodium ascorbate. Dithiothreitol is a dithiol containing reductant with a redox potential of -330 mV (Mathews et al., 1999). Although the redox potential of dithiothreitol is much more negative than sodium ascorbate, the effect is very similar. In the absence of an electron shuttle, no perchlorate is reduced. The only dyes that can catalyze perchlorate reduction are MB and PMS. Again PMS appears to be the more efficient electron shuttle catalyzing the reduction of over 90% of the perchlorate present.
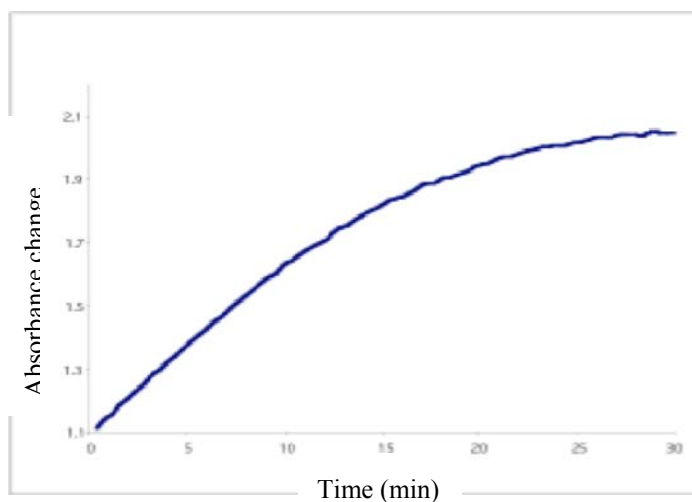
Figure 21. Absorption changes at 614 nm v's time representing methylene blue oxidation by perchlorate reductase in the presence of perchlorate

In figure 20 another dye experiment was conducted, only this time sodium dithionite is used as a reductant. Dithionite is the most reducing of the reductants used containing a redox potential of -540 mV (Mathews et al., 1999). Similar to the previous two experiments mentioned, in the absence of an electron shuttle there is no electron transfer from the reductant to perchlorate reductase (first from left). However, all the dyes tested in the
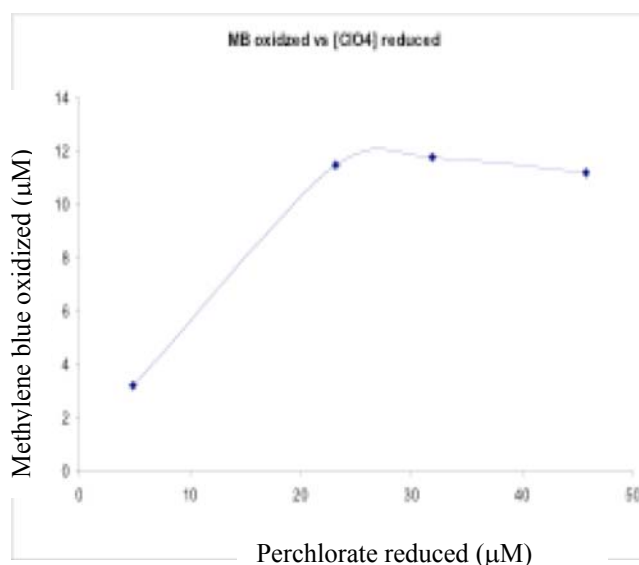
Figure 22. Methylene blue oxidized by perchlorate reductase in the presence of various concentrations of perchlorate

presence of dithionite were able to shuttle electrons to the enzyme. Again PMS (fourth from the left) was the most efficient electron transfer agent, but significant electron shuttling was also seen with AQDS (second from the right).

Based on the above findings, assays were developed with both PMS and MB. MB especially attracted attention because of its intense blue color upon oxidation. In order to make the assay aerobic, we wanted to use a reductant that was resistant to oxidation by molecular oxygen. As shown above, ascorbic acid, a mildly oxygen labile chemical, can reduce methylene blue (although not entirely) and catalyze the reduction of perchlorate through PCR. As can be seen in figure 21, when PCR is incubated with 100 µM MB, 75 µM perchlorate, and 1 mM ascorbate a steady increase at 614 nm is observable. This increase corresponds to the oxidation of methylene blue. However, no linear correlation was found when various concentrations of perchlorate were incubated with 100 µM MB and 1 mM ascorbate (Fig. 22). Therefore assay development with MB was abandoned in order to construct a method using PMS.

PMS is known to be able to oxidize a wide variety of potential reductants that could drive the reduction of perchlorate such as: ascorbic acid, dithiothreitol, NADH, and dithionite. As shown above, three of these four electron donors can catalyze the reduction of perchlorate in the presence of perchlorate reductase. Unlike the reductants tested above, NADH is entirely stable in the presence of oxygen. In addition, NADH has a well-established molar extinction

Absorption change at 340 nm in the presence and absence of perchlorate
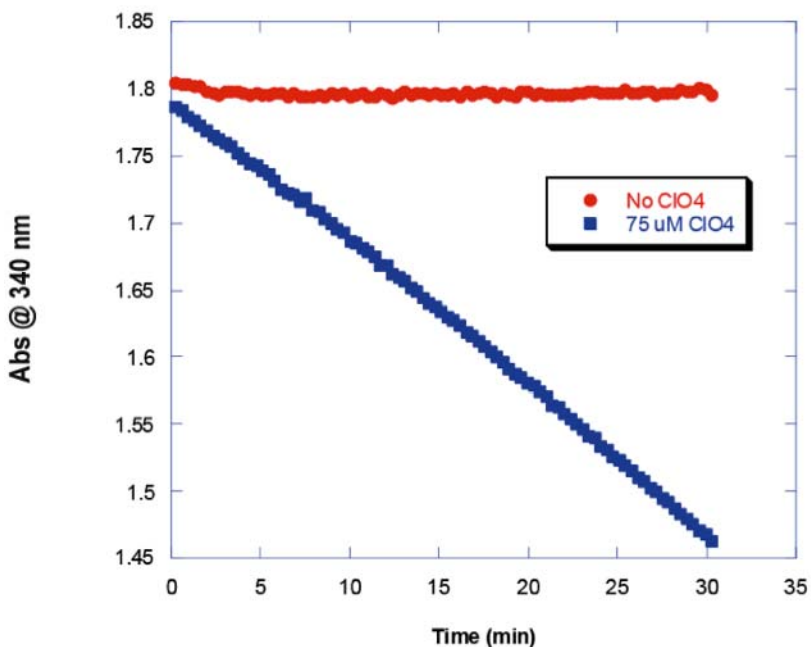
Figure 23. Absorbance changes at 340 nm correspond to NADH being oxidized in the presence of perchlorate. The samples contain 100 µM PMS, 300 µM NADH, 45 µg.ml^{-1} Pcr and indicated values of perchlorate.

coefficient at 340 nm. These characteristics make this chemical perfect for use in our assay. To prove NADH was a suitable reductant, PCR was incubated with NADH, perchlorate, and PMS for 1 hour. Absorbance changes were detected at 340 nm (Fig. 23), and these changes corresponded to a decrease in perchlorate concentration (Fig. 24).

As mentioned earlier, perchlorate is reduced by PCR to chlorite by a four electron transfer, or an oxidation of 2 molecules of NADH. Following the reduction of perchlorate (verified by ion chromatography), it is expected chlorite will react with PMS and NADH. However, as seen in figure 24 this is not the case. As determined from the slope the correlation between NADH molecules oxidized and perchlorate molecules reduced is 2.5:1. This indicates that after perchlorate is reduced by PCR the resulting chlorite molecules only partially react with PMS. Although the lowest concentration of perchlorate tested in these
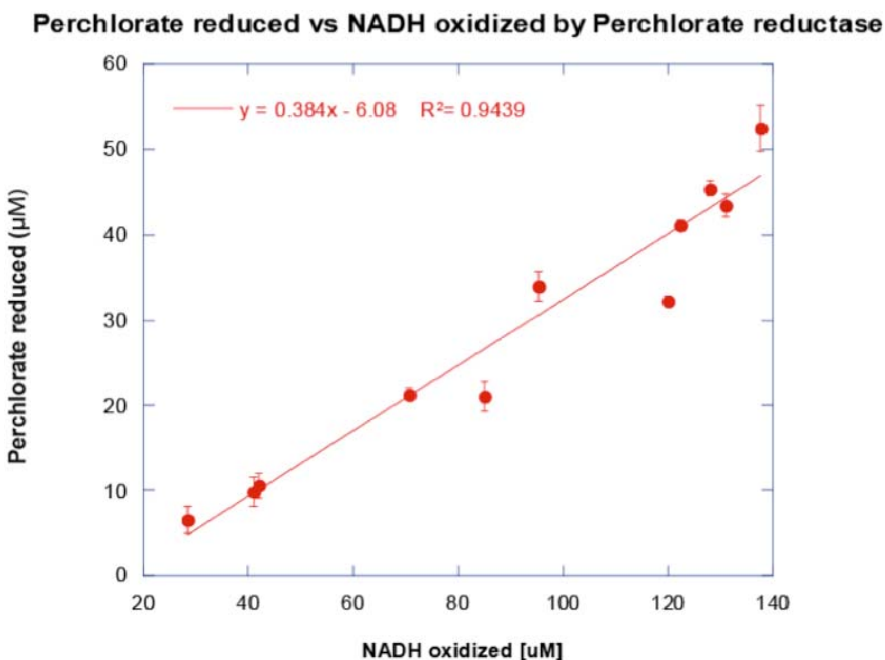
Figure 24. Anaerobic colorimetric bioassay for perchlorate. The indicated amounts of perchlorate were incubated with 100 μM PMS, 300 μM NADH, and 45 μg.ml^{-1} Pcr in a Coy anaerobic glovebag. Perchlorate concentrations were determined using a Dionex ICS 1500 ion chromatograph. NADH concentrations were determined by monitoring absorption changes at 340 nm.

experiments was 5 μM, from our figure it appears 1 μM perchlorate should be readily detectable. However, as previously shown, increasing the temperature from 20° C to 50° C increases the rate of the reaction 1.6 fold. This increase in rate could be due to the reductant in the assay reacting more quickly with chlorite. Therefore, further experimentation is required to determine if temperature could increase the amount of NADH oxidized per molecule of perchlorate reduced. If this is the case, it may decrease the detection limit of the assay further.

Another modification that could lower the detection limit of the assay would be to monitor fluorescence instead of absorbance. As previously published (Held, 2006) by monitoring fluorescence instead of absorbance the detection limit for NADH can be decreased ~26 fold. Studying fluorescence also has the added advantage that it should not be affected by any background in the same way as absorption.

In conclusion, our studies with various dyes have identified a dye-reductant system that has a strong reproducible correlation between perchlorate reduced and a colorimetric change. Although the lowest concentration tested was 5 µM, it appears from figure 24 that the assay should be able to detect concentrations of perchlorate as low as 1 µM (100 ppb). This value is ~17 times higher than the recommended drinking limit for California. However, additional optimizations of the assay, including varying temperature and studying fluorescence instead of absorbance, should decrease the detection limit of the assay to an acceptable range.

Development of an Aerobic assay for perchlorate

Because of the oxygen sensitivity of the Pcr enzyme any assay that determines perchlorate concentrations in the field will require an oxygen removal step. Our first strategy to alleviate the effect of oxygen was to reduce it with a reductant in the

Figure 25. Dithiotreitol inhibits oxidation of NADH by Pcr in the presence of 100 uM perchlorate

reaction mixture. From observation, it was discovered that PMS oxidized NADH much more quickly than it oxidized thiols. Experiments were conducted to examine the effect of dithiothreitol (DTT) and cysteine (CYS) on the rate of NADH oxidation by PCR in the presence of 100 µM perchlorate. As can be seen in figure 25, under anaerobic conditions 0.5 mM, 1 mM,

2 mM, 4 mM, and 8 mM DTT inhibited the NADH oxidation by 32%, 32%, 38%, 59%, and 87%, respectively. This inhibition would be hard to overcome in a practical assay, so the idea of using DTT as an antioxidant in the reaction was abandoned.
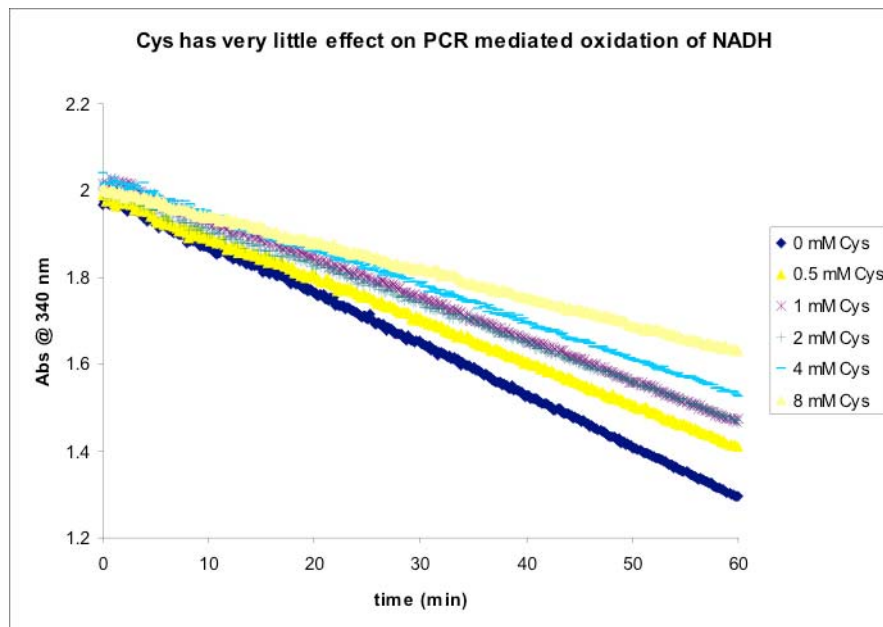
Figure 26. Effect of cysteine on the oxidation of NADH by Pcr in the presence of 100 μM perchlorate.
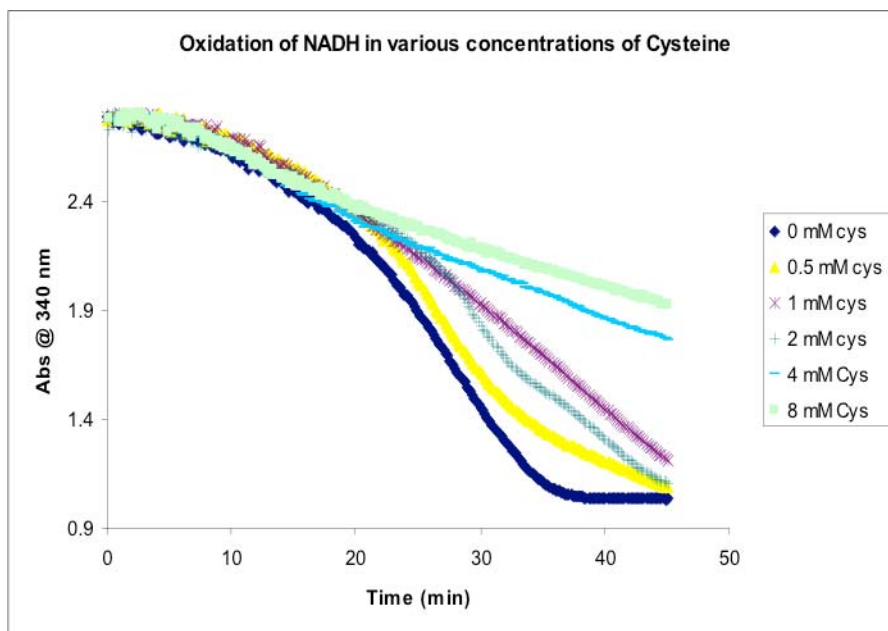
Figure 27. Cysteine cannot prevent the abiotic oxidation of NADH in the bioassay reaction system by molecular oxygen.

As shown in figure 26, cysteine had less of an inhibitory effect on the reaction. In one hour 0.5 mM, 1 mM, 2 mM, 4 mM, and 8 mM cysteine inhibited the NADH oxidation by 17%, 24%, 24%, 32%, and 48%.   Although this inhibition is significant, these same concentrations of cysteine were added to NADH and PMS under aerobic conditions in order to see if this cysteine could inhibit the abiotic oxidation of NADH by molecular oxygen (no perchlorate present). As can be seen in figure 27, no concentration of cysteine was able to prevent a rapid oxidation of NADH by oxygen. As seen in figure 27 (blue), after 45 minutes of incubation with 8 mM cysteine (the highest concentration added to the solutions) 34% of the NADH in the solution was oxidized by molecular oxygen. The experiments shown above disproved the hypothesis that antioxidants could be added to the reaction to quench molecular oxygen during the quantification of perchlorate.

In an attempt to prevent oxygen from entering the assay mixture, mineral oil was layered on top of the reaction solution in a glass cuvette. Mineral oil was selected as a "capping" solution because of its low oxygen solubility and cost. In addition, anaerobic microorganisms have been cultured in anaerobic media underneath mineral oil, thus proving its usefulness in such a
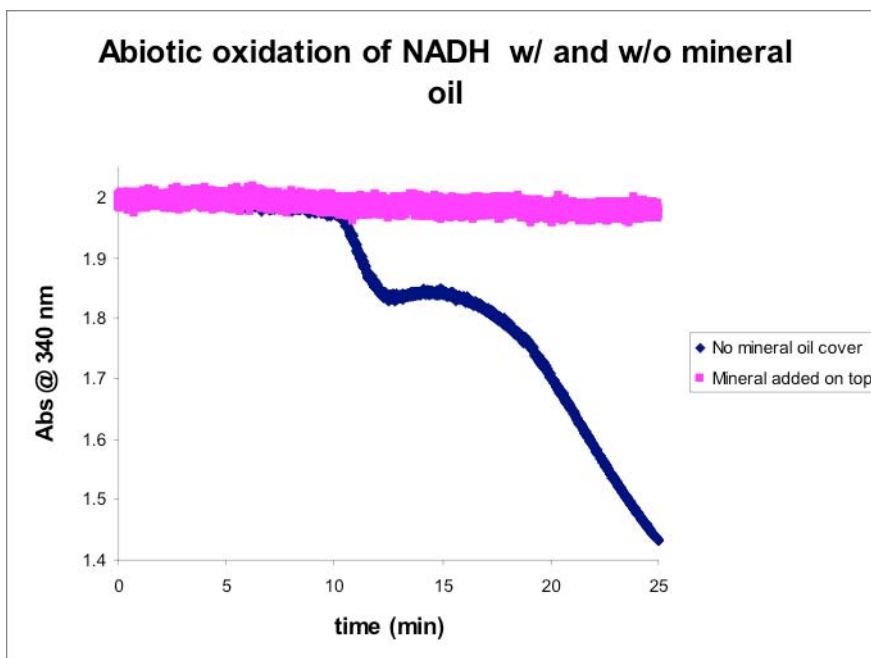
Figure 27. Mineral oil prevents the abiotic oxidation of NADH by oxygen

situation (Little and Subbarow, 1945). As shown in figure 27 mineral oil prevents the oxidation of NADH over the coarse of 25 minutes. In comparison to the control (no mineral oil) the sample is quite resistant to oxygen diffusion, and abiotic NADH oxidation.

Although, mineral oil does prevent the diffusion of oxygen into the cuvette, there is still oxygen in the original solution. This oxygen has to be reduced before the reaction with PCR can occur. As mentioned earlier oxygen can denature PCR, and it can oxidize PMS resulting in a large background that could affect perchlorate quantification. In addition, adding competing reductants to reduce the oxygen could interfere with the assay.

Based on the characteristics of NADH and PMS, a protocol was developed that could reduce all of the oxygen in the solution without using additional chemicals that could interfere with the assay. First, we added the mineral oil to the top of the solution in a 1 ml quartz cuvette. The cuvette cannot be plastic because oxygen can readily diffuse through plastic to contaminate the solution. The mineral oil partitions on top of the solution, and because of its viscosity and hydrophobic nature, does not mix with the aqueous layer that contains the perchlorate. Underneath the mineral oil, PMS is added to the aqueous phase (final concentration 175 µM). The solution takes on a yellowish color when the PMS is oxidized (figure 28, right cuvette).

Following the addition of PMS, NADH is added to the solution underneath the mineral oil (final concentration 1 mM). Once NADH is added to the aqueous solution, PMS is rapidly reduced to its hydroquinone form. This molecule quickly reduces all of the oxygen in the cuvette. Once this process is completed, the aqueous solution turns clear (figure 28, left). Following this colorimetric change, the solution is essentially anaerobic and the enzyme can be added. The enzyme itself is

Figure 28. Mineral oil prevents the abiotic oxidation of NADH in the bioassay system by molecular oxygen

stored in a vessel covered in mineral oil. NADH and PMS are added to solution to keep it anaerobic. The enzyme is added underneath the mineral oil to maintain anaerobicity. After a brief mixing period using a micropippetor, an absorbance is taken of the solution at 340 nm. Following an incubation of one hour, a second absorbance is taken. The difference in the two measurements corresponds to the amount of NADH oxidized by PCR to reduce perchlorate (more below).

However, we found one additional problem with the set-up of our assay. Oxygen would slowly diffuse into the solution from the walls of the cuvette, and through the mineral oil. This oxygen contamination was small, but was not precisely reproducible leading to a large error in our measurements. We found that by increasing the viscosity of the solution, we could prevent oxygen diffusion and abiotic oxidation of NADH. As shown in figure 29, abiotic oxidation of NADH in a glycerol free solution (in pink) is slow, resulting in an oxidation of ~12 µM NADH in 6 minutes. This is a mere 4% of the NADH in the cuvette, but the error created by this value could hinder the detection of small quantities of perchlorate. When the glycerol concentration is increase to 5%, the amount of NADH oxidized by molecular oxygen decreases to 7.5 µM in the time scale observed (Fig. 29 yellow). Once the glycerol concentration is increased to 6%, the NADH oxidized by oxygen in the time framed observed decreases to 0% (Fig. 29, blue). This effect is reproducible, and three standard curves of perchlorate reduced vs. NADH oxidized were generated in the presence of 6% glycerol using the above protocol (Fig. 30). Judging by the slopes of these curves it appears the enzyme is still oxidizing 2.5 molecules of NADH per molecule of perchlorate. The y-intercepts of these curves are significantly different, but this difference could correspond to different temperatures or small amounts of oxygen that diffused into the cuvette during the experiment. These results stress the need to analyze all potential perchlorate containing samples alongside a blank that contains no perchlorate. This blank would help account for all abiotic reactions that contribute to any background signal.
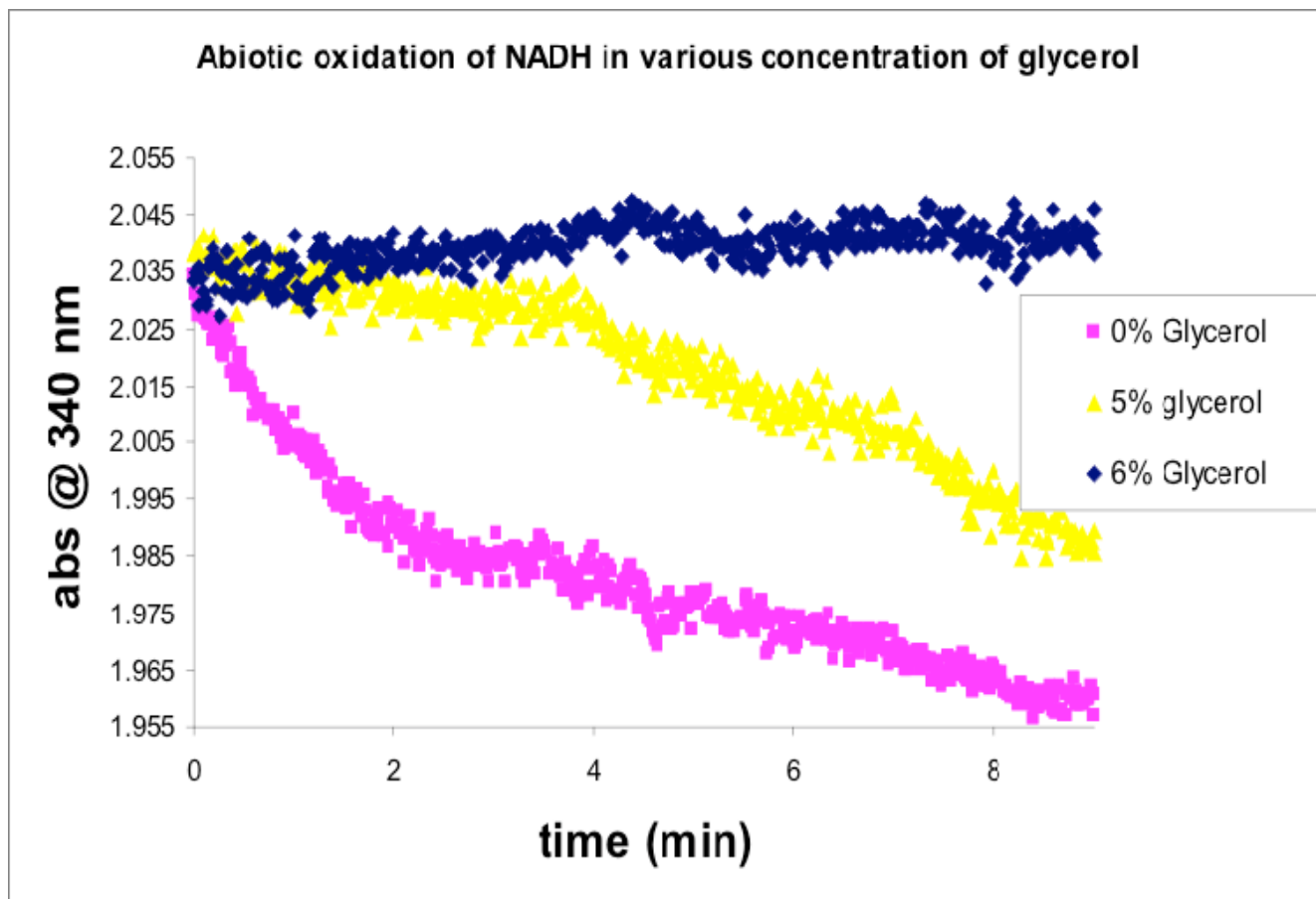
Figure 29. Glycerol increases the viscosity of solution thus preventing a slow abiotic oxidation of NADH in the bioassay system from residual oxygen diffusing from the cuvette walls.
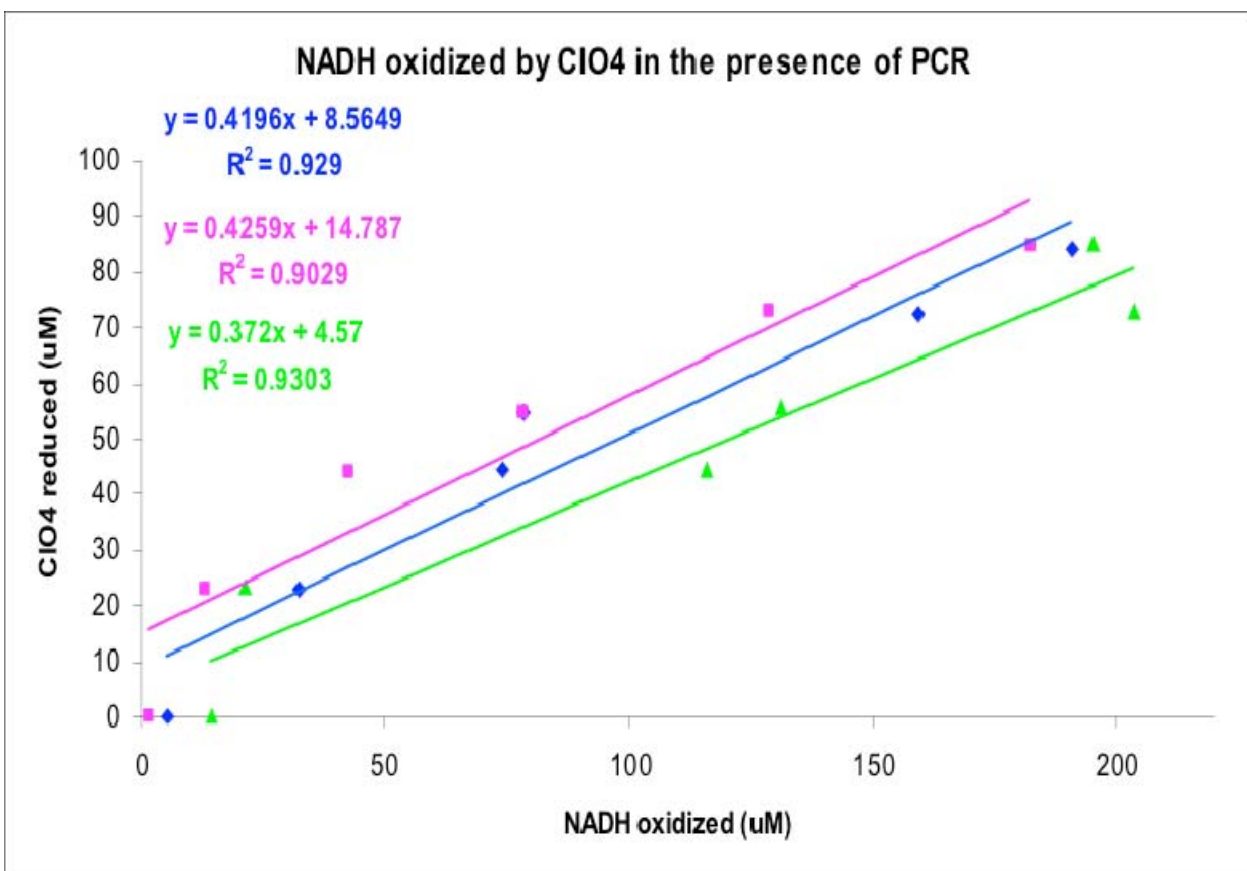
Figure 30. Aerobic assay to determine perchlorate concentration using glycerol to increase viscosity. A series of concentrations were analyzed in three trials. The data points, as well as the fits for each trial are shown in different colors. The lowest concentration analyzed with this assay was 5 μM perchlorate.

Alternatively we tried replacing glycerol with sodium chloride as the viscosity increasing agent. Sodium chloride is potentially a better chemical to use to decrease oxygen diffusion, as salinity can affect oxygen solubility in solution. In addition, heating the solution decreases oxygen solubility making increases in temperature a possibility for later
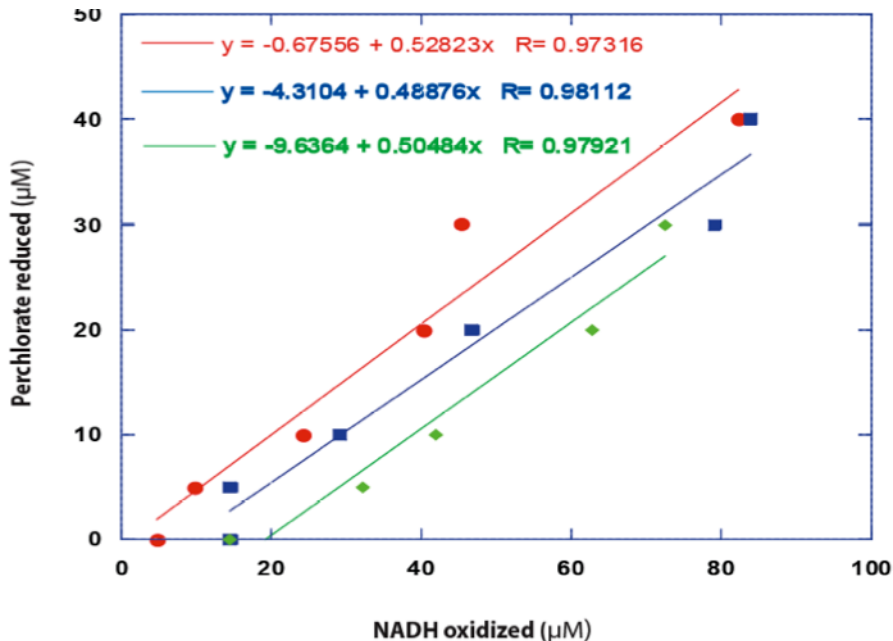
Figure 31. Aerobic assay to determine perchlorate concentration using sodium chloride to increase viscosity and decrease oxygen solubility. A series of perchlorate concentrations were analyzed in three separate trials. The datapoints as well as the fits for each trial are shown in different colors. The lowest concentration analyzed

analyses. In figure 31, three standard curves were generated with various concentrations of perchlorate using the protocol outlined above. Sodium chloride (2 M) was added to the solution instead of glycerol to decrease oxygen diffusion from the walls of the cuvette, and through the mineral oil. As can be seen (Fig. 31) when sodium chloride is used instead of glycerol the results are more reproducible, and the detection limit appears to be smaller.

Optimizing the purification of PCR:
The purification of PCR was done as previously published (Kengen et al., 1999) with the following modifications (outlined below). These modifications were carried out so that the purification could be done in a shorter span of time. Overall this purification took about 16 hours (two days). This decreases the time required for this procedure by 60%. Although this is still a significant period of time, the revised protocol is easier and quicker.

Cells were lysed in an anaerobic chamber by sonication following a brief lysozyme treatment. Twenty grams of cells were dissolved in 100 ml of MOPS buffer (pH 7). The buffer also contained sodium dithionite (1 mM), phenylmethylsulfonyl fluoride (0.5 mM), and Dnase (10 µg/ml). Lysozyme was added to the solution at room temperature to a final concentration of 0.1 mg/ml. Following the addition of lysozyme, the cell solution was allowed to stir in the anaerobic chamber for 30 min. After this treatment, the cells were sonicated using a 550 Fisher scientific sonic dismembrator. Cells were sonicated in 500 ms pulses, followed by 500 ms pauses, for 2 minutes. This process was repeated twice.

Following sonication, membranes and unbroken cells were spun down in a Beckman ultracentrifuge. The cell lysate was placed in 25 ml polypropylene tubes and spun in a Beckman TI-50 centrifuge at 35,000 RPM for 1 hour. The supernatant was retained, and the pellet was discarded. Glycerol was added to the supernatant to a final concentration of 8%. Following the addition of glycerol the samples were aliquoted into 5 ml fractions and flash frozen in liquid nitrogen.

For purification, the soluble lysate was first loaded onto a DEAE anion exchange column. The DEAE column was equilibrated in 25 mM Trishydroxmethylamine buffer with 1 mM sodium dithionite (pH 8.5). Neither PCR nor chlorite dismutase bind to the resin, and both elute in the flow thru. The phosphate concentration in this partially purified sample was brought to 150 mM (1 mM dithionite), and it was incubated with 4 grams ceramic hydroxyappetite in a 45 ml oakridge tube (final volume 30 mL). The tubes were placed in an icebox on top of a platform shaker set on low for gentle agitation for 30 minutes. Following this incubation the resin was spun out of the solution at 2,000 x g. The supernatant was discarded, and the resin was washed 2 times with 150 mM phosphate (1 mM dithonite). The protein was eluted from the resin with 10 ml of 450 mM phosphate (1 mM dithonite). Following a 30 minute incubation on a shaker platform at 4 C, the resin was removed from the supernatant through centrifugation (2,000 x g). This elution was repeated with 10 ml of 450 mM Phosphate (1 mM dithonite). The two 10 ml fractions were pooled and the ammonium sulfate concentration was brought to 90% saturation. The solution was placed in the anaerobic chamber and stirred on ice for 20 minutes. Then, the solution was spun down at 12,000 x g and 4 C. The supernatant was discarded and the

precipitated protein was dissolved in 2 ml of 50 mM MOPS (1 mM dithionite). The sample was then loaded onto a gel filtration column (sephacryl S-600, pharmacia). The sample partitions into two bands, and both were collected by visualization.

Table 1 – <u>Activity analysis of the purified protein</u> Activity was determined under anaerobic condition using the colorimetric bioassay.

Step	Volume (ml)	[Protein] (mg/ml)	Total Activity (U)	Sp Act (U/mg)	Yield (%)	Fold purification
Soluble enzymes	23	14.47	6321	19	100%	1
DEAE	30	2.936	4353	49	68%	2.60
Hydroxyapetite $(NH_4)_2SO_4$ cut	2	4.21	1241	174	19%	7.75
Gel filtration (65-85)	18	0.394	2782	374	44%	19.70

Table 1 shows the purification results in a 19.7 fold purification. This is similar to previously published results for the purification of PCR (Kengen et al., 1999). The specific activity is given in units. A unit is the amount of enzyme required to oxidize 1 µM of ClO_4/ min/ mg protein. The amount of ClO_4 reduced is estimated by the amount of NADH that is oxidized. For the first two samples (soluble enzymes and DEAE), it is considered that 4 molecules of NADH are oxidized per molecule of ClO_4. In the last two samples, two molecules of NADH are assumed oxidized per molecule of ClO_4. A similar technique is used to treat the data in Kengen et al (Kengen et al., 1999).

The gels shown (Fig. 32) demonstrate that the final sample is pure, containing only two polypeptides (PcrA and PcrB). The yield of this purification appears to be approximately 3 times the yield in Kengen et al., however, it is unlikely that this value is true. What is more likely is that the amount of PCR in the soluble enzymes is an amount outside the linear relationship between concentration of enzyme and activity. This also explains the low yield in the Hydroxyappetite fraction, but high yield in the final samples.

A. Lysate (10 ug)
B. After DEAE column (10 ug)
C. After Hydroxyappetite column ((NH4)2SO4 cut) (10 ug)
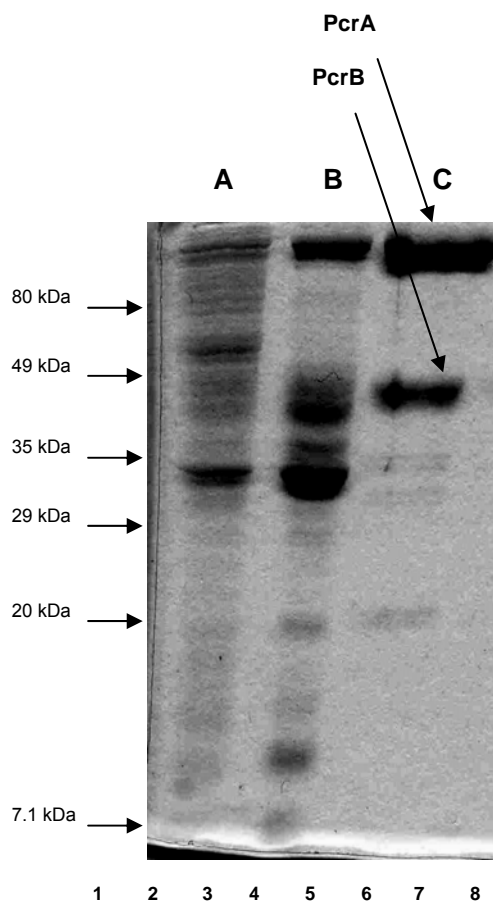
1. After Hydroxyappetite column ((NH4)2SO4 cut) (2 ug)
2. Gel filtration 25-35 ml (2 ug)
3. Gel filtration 35-45 ml (2 ug)
4. Gel filtration 45-55 ml (2 ug)
5. Gel filtration 55-65 ml (2 ug)
6. Gel filtration 65-75 ml (2 ug)
7. Gel filtration 75-85 ml (2 ug)
8. Gel filtration 85-95 ml (2 ug)
9. Gel filtration 95-105 ml (2 ug)
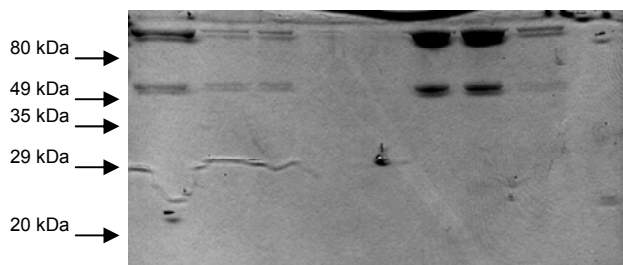
Figure 32. Purification of Perchlorate reductase using a streamlined protocol: Samples were analyzed on a 12% acrylamide gel, and stained using Coomassie G-250.

Analysis of perchlorate using whole cell lysate

An alternative to using the purified enzyme in this assay is to use the whole cell lystate from

Dechloromonas agitata str CKB. Unlike purified enzyme this product can be acquired quickly (~2 hrs). In addition, whole cell lysate oxidized more NADH per molecule perchlorate, translating to an increase in signal intensity. The increase in signal intensity is because of chlorite dismutase. It transforms chlorite into chloride and oxygen, which quickly reacts with reduced PMS. Compared to molecular oxygen, chlorite reacts slowly with the reduced shuttle.

NADH oxidation vs perchlorate concentration in the presence of CKB lysate

$y = 3.8952x - 5.332$
$R^2 = 0.9941$
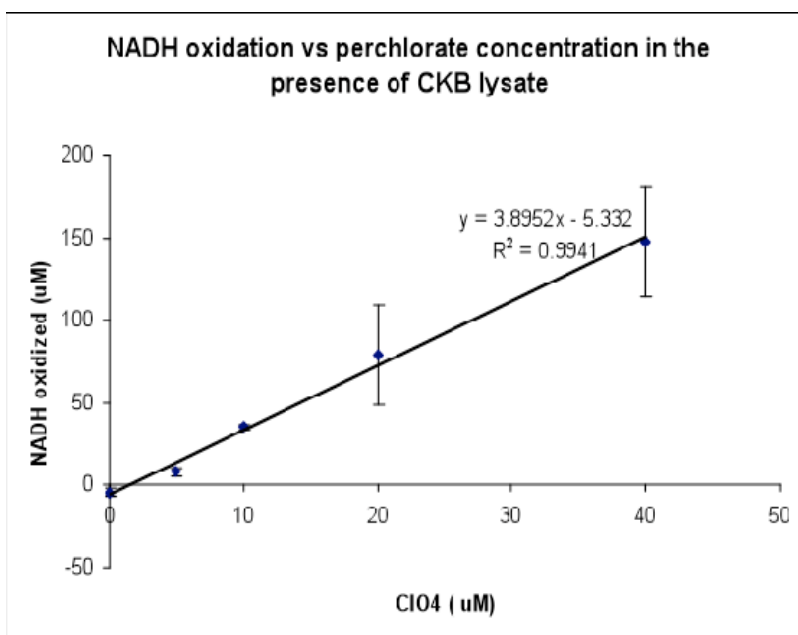
Figure 33. Whole cell lysate can replace perchlorate reductase in the bioassay.

In order to quantify perchlorate in solution, 3 uL of a 33 mg/mL protein solution (CKB lysate) was added to a 300 uL solution containing between 0 and 40 uM ClO_4 (50 mM MOPS). PMS solution was added to 175 µM and NADH concentration was brought to ~320 µM. The reactions were carried out in the anaerobic chamber at room temperature.

As can be seen in figure 33, there is a linear correlation between the amount of perchlorate in the sample and the amount of NADH oxidized ($R^2 = 0.9941$). However, several other interesting observations can be made. First, the slope gives a correlation between the NADH oxidized and the perchlorate reduced of 3.89 NADH oxidized/ ClO_4^- reduced. This value roughly correlates with the theoretical amount of NADH that should be oxidized for the reduction of perchlorate to chloride (8 electrons or 4 molecules of NADH). Second, the standard deviations of the higher concentrations (20 and 40 µM) is rather high. This indicates that other reactions are most likely

occurring where NADH is being oxidized or reduced. This is not an improbable hypothesis because there are so many other enzymes in this solution.

In order to stop these secondary processes, 2 M NaCl has been added to the solution. Salt addition will need to be done in the aerobic assay to prevent oxygen diffusion into the solution. This increase in salinity can also decrease the activity of many enzymes, but has only limited effect on perchlorate reductase and chlorite dismutase. As expected, the linear correlation between perchlorate concentration and NADH oxidation is unaffected by the presence of 2 M NaCl (Fig. 34).
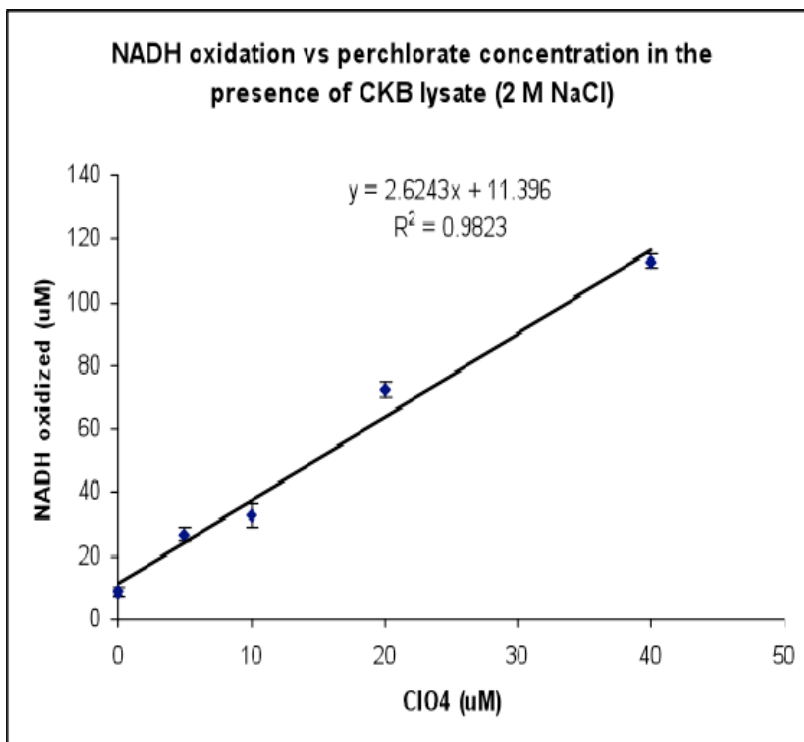
Figure 34. Sodium chloride reduces the standard deviation of the bioassay when whole cell lysate is the catalyst.

Interestingly, the stoichiometric relationship between NADH oxidized and perchlorate reduced has changed (2.6 molecules NADH oxidized/ ClO_4^-). This difference could be attributed to a slower reaction rate for the lysate in the presence of high salinity. This problem can be easily solved by adding more lysate or giving the reaction more time.

Set up of Thorne assay:
The developed bioassay can only detect perchlorate at concentrations of 100 ppb (1 μM). Therefore, a concentration step is needed to allow detection of perchlorate in environmental samples below federal and state guidelines (e.g. regulatory limit 6 ppb or 0.06 μM for California). The method we have adapted was initially developed by Philip Thorne and published in a US Army Corps of Engineers report (Thorne, 2004).

In this assay (later referred to as the Thorne assay), decyltrimethyl ammonium bromide (DTAB) is added to a styrene divinylbenzene (SDVB) column. Because of DTAB's hydrophobic ten-carbon tail it can stick to the SDVB column. However, the molecule is positively charged, and therefore can bind perchlorate in solutions with a concentration as low as 0.5 ppb. The DTAB-perchlorate ion pair can then be eluted using acetone, which weakens the interaction of DTAB with the SDVB columns.

Figure 35. Millipore ultrafiltration device: A vacuum line was used to pull liquid through SDVB columns (in green stoppers). The liquid was collected in the bottom of the device and removed the black plug at the bottom of the apparatus.

Trouble Shooting and Optimizing the Thorne Assay

In order to analyze multiple samples quickly, we have set SDVB columns in a Millipore vacuum manifold set up (Fig. 35). Using this apparatus, twelve samples can be analyzed simultaneously. The concentration of twelve 500 mL samples requires about 30 minutes. The perchlorate in these samples is subsequently eluted using acetone. This acetone is evaporated by heating at 55 – 70° C. The evaporated sample is dissolved in ddH$_2$O and analyzed using the bioassay as well as ion chromatography.

Although initially this set-up proved to be a functional method for perchlorate detection in small sample volumes, larger sample volumes did not concentrate and elute consistently (Fig. 36). We found that the larger the sample volume, the less perchlorate bound and eluted from the column. One possible explanation for this phenomenon is that the liquid does not pass equally through the column resin. This could create tiny streams within the resin that allows perchlorate to flow through the resin without contacting the DTAB molecules. However, the original protocol for this technique only used pressure to move liquid through the columns.
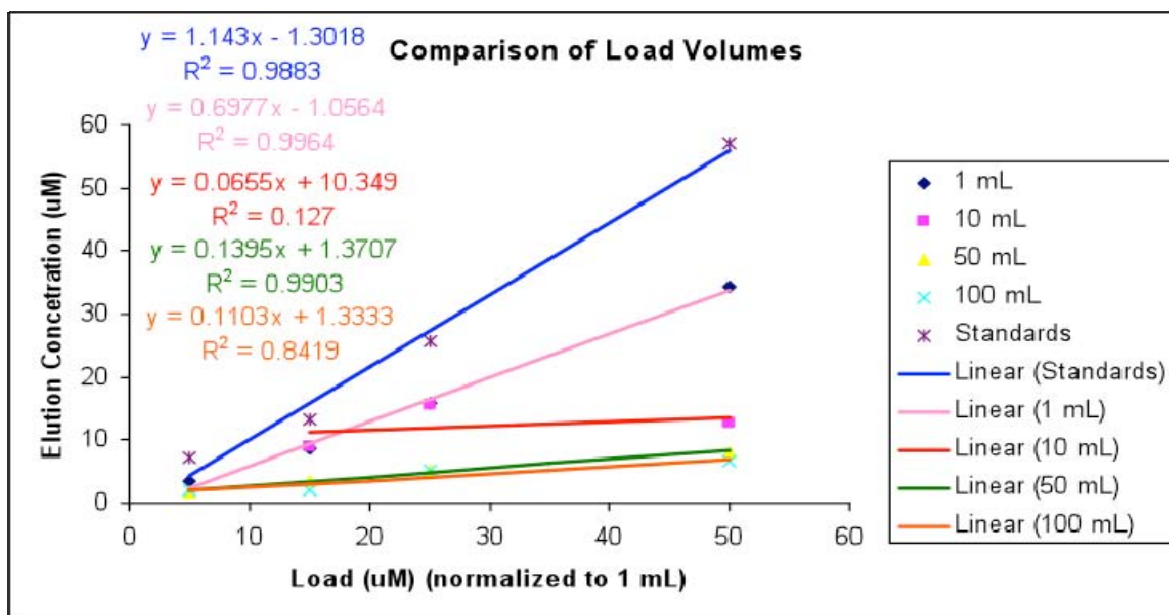
Figure 36. SDVB columns bind a smaller percentage of perchlorate under vacuum. One milliliter of the concentrations shown on the x-axis was diluted in the volumes shown in the legend. As can be seen below, as the volume of the sample increases the amount of perchlorate that binds and elutes from the columns decreases.
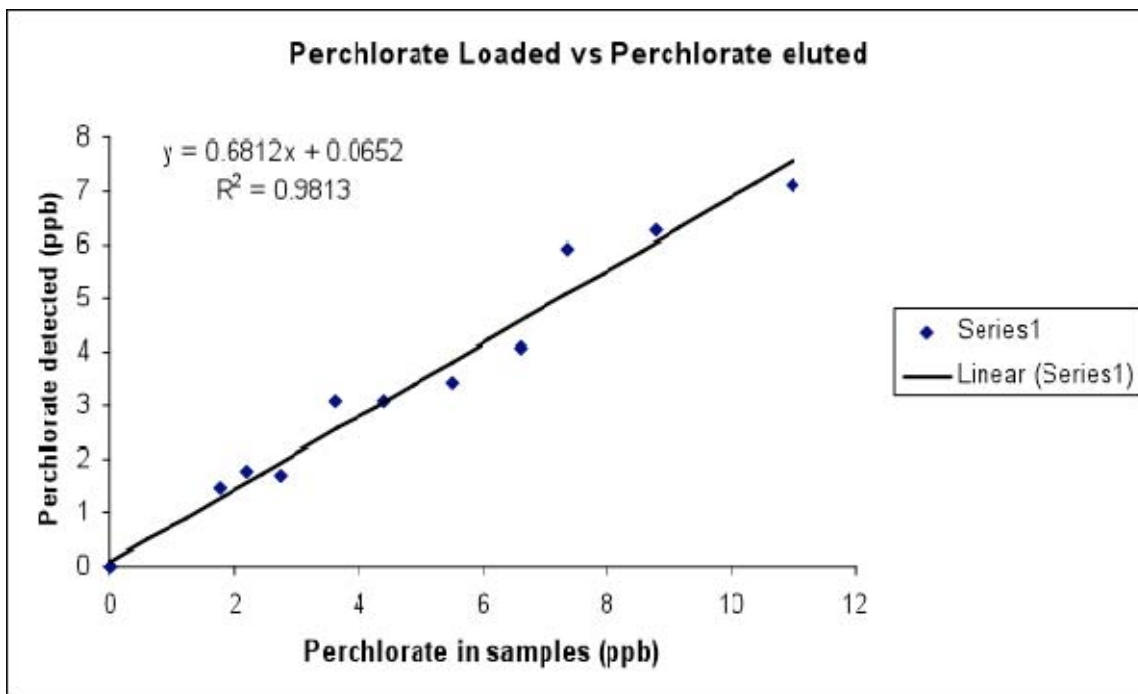
Figure 37. SDVB columns bind and elute perchlorate under pressure: 100 mL samples of distilled water were spiked with perchlorate and concentrated on SPE columns using the Thorne protocol. The perchlorate that eluted from the columns was analyzed using ion chromotography. Approximately 70% of the perchlorate that was loaded eluted with 1 mL of acetone.

To counteract this problem, 200 mL of perchlorate containing samples were pushed through SDVB columns using 60 mL syringes. As determined by ion chromatography, 70% of the perchlorate that was bound to the columns eluted with 2 mL of acetone (perchlorate detected following the evaporation of acetone). This seemed to suggest that liquid could not be vacuumed through the column, but it could be pushed through the column using pressure (Fig. 37).

Although we found that acetone eluted perchlorate from our columns, these samples could not be analyzed by the bioassay. Even with heating to a temperature of 90 C to remove residual acetone, there was no reliable linear correlation between the perchlorate in the samples and the NADH oxidized in the bioassay (Fig. 38). It is possible that residual acetone is interfering with the assay, or upon heating DTAB produces a side product that inhibits the enzyme.

Because of the inhibition caused by the acetone/DTAB, it was decided to modify the method of elution. Solutions of various alternative anions that would displace perchlorate on the column
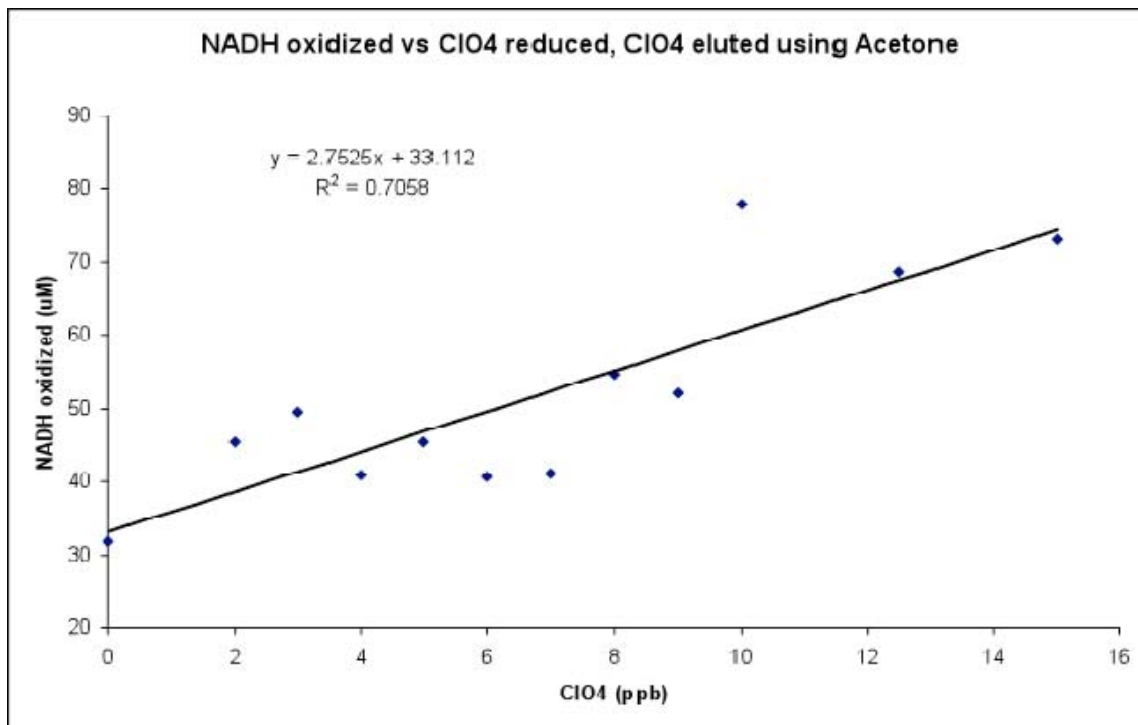
Figure 38. Acetone interferes with the bioassay: Although samples were boiled to remove acetone after the elution, there appears to be some residual contaminant that interferes with the bioassay.

were tested as an eluant instead of adding acetone, which elutes DTAB and perchlorate as an ion pair. Triplicate SDVB columns were loaded with 100 mL of 10 ppb perchlorate. The perchlorate was eluted with various ions and compared to elutions with acetone (Fig. 39). Iodide ion elutes perchlorate with an efficiency comparable to acetone. This makes sense as iodide has a similar ionic radius and solvation energy when compared to perchlorate (Brown and Gu, 2006). In addition, many other ions were found to elute perchlorate. Morpholinopropane sulfonic acid (MOPS) was found to successfully elute perchlorate from the SDVB columns, however the amount it could elute was pH dependent. When the pH was raised from 7 to 13 the amount of perchlorate that eluted from the SDVB columns with 200 mM MOPS increased from 7% to 58% (Fig. 39). The ability of MOPS to elute perchlorate was interesting as it is a necessary component of our bioassay. In addition, 2 M chloride was capable of eluting small amounts of perchlorate, but sulfate, phosphate, iodate, and SDS eluted little to no perchlorate.

As iodide was found to elute as much perchlorate as acetone, we decided to try and elute perchlorate from SDVB columns using iodine and analyze these samples using the bioassay.
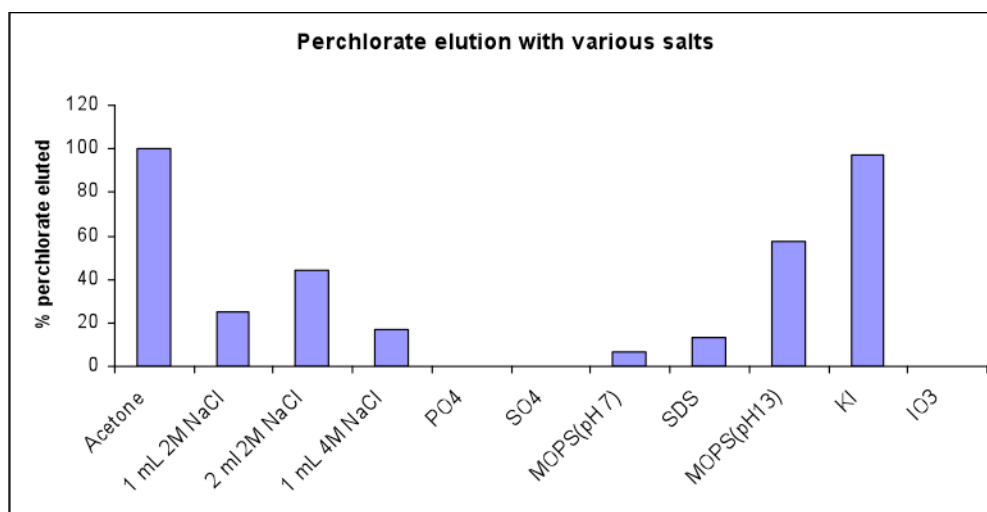
Figure 39. Elution of perchlorate from SDVB columns with various anions: In order to adapt the Thorne assay to our bioassay, ionic substances were tested as potential elutants. All salts shown below have a concentration of 500 mM except sodium chloride (2 M). As can be seen, MOPS (sodium morpholinopropane sulfonic acid) elutes 57% of the perchlorate from the SDVB columns. This value can be increased to 100% if the volume of MOPS is double the column volume. In addition, MOPS is a gentle biological buffer that has no deleterious effects on our assay. The pKa of MOPS (~7) is close to the optimum pH of perchlorate reductase.

The results indicated that there was no linear correlation between the perchlorate in the samples (0-20 ppb) and the NADH oxidized by whole cell lysate (Fig. 40). The perchlorate was verified to be present using ion chromatography suggesting that iodide was interfering with the bioassay, and could not be used to elute perchlorate.

Because iodide inhibited perchlorate reductase, MOPS was investigated as a potential eluant. 1 mL of 200 mM MOPS at pH 13 was capable of eluting 58% of the perchlorate bound to the columns, however 2 mL of 200 mM MOPS was capable of eluting almost 100% of the perchlorate from the columns. Although this anion is an essential component in the bioassay to maintain pH, the sample must be pH neutralized before it can be
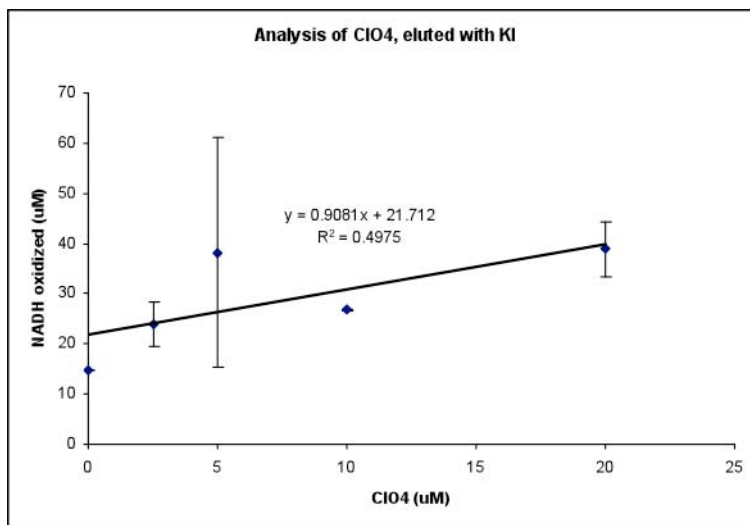
Figure 40. Bioassay done following elution with potassium iodide: 100 mL samples were concentrated on the SDVB columns and eluted with 25 mM potassium iodide. This ion had a strong affect on the linearity of the bioassay.

analyzed using the bioassay. Therefore, following the elution of perchlorate from the columns using MOPS buffer, the samples were neutralized using 1 M HCl (final pH 7.5, checked by pH paper). A linear correlation was observed between the amount of perchlorate in the original samples and the amount detected by the bioassay (Fig. 42). The R^2 value relating the two is 0.88, a value that should be high enough for a field analysis. The average standard deviation for the samples is 2 ppb, an error that should be small enough for our assay. In addition, based on the y-intercept the assay appears to have a lower detection limit of 2 ppb, well below California's legal limit of 6 ppb.

Detecting perchlorate in the presence of various groundwater contaminants: Purifying perchlorate from anionic contaminants

Our results demonstrate that perchlorate reductase can also react with nitrate and chlorate as such it is imperative that these compounds be removed from any environmental sample prior to

perchlorate analysis. As previously shown, these ions can also bind to DTAB when it is ligated to a SDVB column, and co-elute with perchlorate.   However, nitrate and chlorate can be separated from perchlorate with a wash of 2.5 mM DTAB and 15% acetone (in a 1 mL column). This wash can remove up to 100 ppm nitrate and 1 ppm chlorate without any loss of perchlorate from the SDVB columns.

Table 2 shows various concentrations of perchlorate that were detected in 200 mL samples that contained significant concentrations of nitrate or chlorate.   Each sample was analyzed in triplicate, and shown below are the averages of each analysis. As mentioned above, samples were loaded onto SDVB columns that were preconditioned with DTAB. Following the wash with 2.5

Figure 42.   Perchlorate detected using MOPS to elute the SDVB columns: 200 mL samples containing the perchlorate concentrations shown on the x-axis were loaded onto SDVB columns and eluted using 200 mM MOPS (pH 12.5). Following neutralization the samples were analyzed using the colorimetric bioassay, and perchlorate concentration were determined using a standard curve.

mM DTAB and 15% acetone, the perchlorate was eluted with 2 M NaCl and 200 mM MOPS (pH 13). Perchlorate was detected using the developed colorimetric bioassay, which uses whole cell lysate from *Dechloromonas agitata* str CKB.   A standard curve for this analysis was generated using a perchlorate standard purchased from Alltech Associates Inc.

Table 2 – <u>Effect of nitrate and chlorate on perchlorate detection using the bioassay</u> Perchlorate values were determined using a standard curve.

Actual Concentration (ppb)	ddH$_2$O (no co-contaminants)	100 ppm Nitrate	1 ppm Chlorate
0	-1.86	1.09	-0.93
5	4.51	3.76	3.82
10	10.53	6.72	6.38
15	14.30	16.35	12.21
20	18.91	21.87	17.95

The results indicate that anionic contaminants did not cause false perchlorate readings, and the bioassay error was still ~2ppb.

Analyzing tapwater, groundwater, and samples of various ionic strengths
In order to determine the effect of various ions on the bioassay, we made solutions of 0, 5, 10, 15, and 20 ppb perchlorate in solutions of ddH$_2$O, 100 ppm ferric iron, 100 ppm chloride, 100 ppm phosphate, 100 ppm nitrate, 100 ppm sulfate and 1 ppm chlorate. The samples were concentrated on SDVB columns that were equilibrated with DTAB. Perchlorate was eluted from the columns using 200 mM MOPS and 2 M NaCl. As was seen by both ion chromatography and the colorimetric bioassay, ionic strength interferes with either the binding or eluting of perchlorate from the column. In table 3, less perchlorate binds/elutes from the columns when 100 ppm chloride and 100 ppm Fe(III) are used in the solution as opposed to the uncontaminated sample. To prove this effect was not due to the bioassay, samples were also analyzed by ion chromatography (table 3).   All ions listed above display a similar effect on perchlorate binding/eluting from the column, indicating that the problem is due to increasing ionic strength rather than an ion-specific reaction. In order to see if the ionic strength effects would hinder the binding/eluting of perchlorate to the resin in tapwater, perchlorate was analyzed in a solution that had the maximum legal limit of many ions found in tapwater. The solution, called extreme tapwater, contained 5.6 mM NaCl, 0.97 mM NaNO$_3$, 0.1 mM Na$_2$SO$_4$, 0.16 mM Na$_2$CO$_3$, 63 uM NaBr, 0.12 uM NaClO$_3$, and 0.078 uM NaBrO$_3$. Perchlorate samples in ddH2O and extreme tapwater were loaded onto SDVB

Table 3 – Effects of ionic strength on the perchlorate detected in ddH$_2$O 200 mL samples were loaded on the SDVB columns and eluted with 2 mL of MOPS buffer. Perchlorate was determined using the bioassay or ion chromatography. Perchlorate concentrations in the 2 mL samples were divided by 100 (dilution factor) to determine the original perchlorate concentration.

No contaminant

ClO4 (μM)	Ion chromatography (ppb)	Bioassay (ppb)
0	0 +/- 0	-1.86 +/- 2.00
5	5.96 +/- 0.35	4.51 +/- 2.12
10	8.17 +/- 0.66	10.53 +/- 3.39
15	12.76 +/- 1.96	14.30 +/- 1.25
20	15.33 +/- 1.25	18.91 +/- 3.76

100 ppm Cl$^-$

ClO4 (μM)	Ion chromatography (ppb)	Bioassay (ppb)
0	0.05 +/- 0.09	-4.73 +/- 1.45
5	1.90 +/- 1.60	-.8894 +/- 1.80
10	5.52 +/- 0.45	3.25 +/- 4.91
15	7.81 +/- 0.99	5.45 +/- 4.05
20	10.96 +/- 0.75	10.67 +/- 4.33

100 ppm Fe(III)

ClO4 (μM)	Ion chromatography (ppb)	Bioassay (ppb)
0	0.13+/- .23	0.58 +/- 3.01
5	2.20 +/- 0.60	3.20 +/- 3.62
10	5.24 +/- 0.18	4.27 +/- 1.22
15	6.06 +/- 5.11	5.14 +/- 5.37
20	9.94 +/- 0.68	10.06 +/- 1.30

columns as previously described. As determined by ion chromotography, the amount of perchlorate that eluted from the columns is less in the extreme tapwater sample than the ddH2O sample (Fig. 43) supporting that ionic strength interfered with the binding and elution of perchlorate from the SDVB columns.
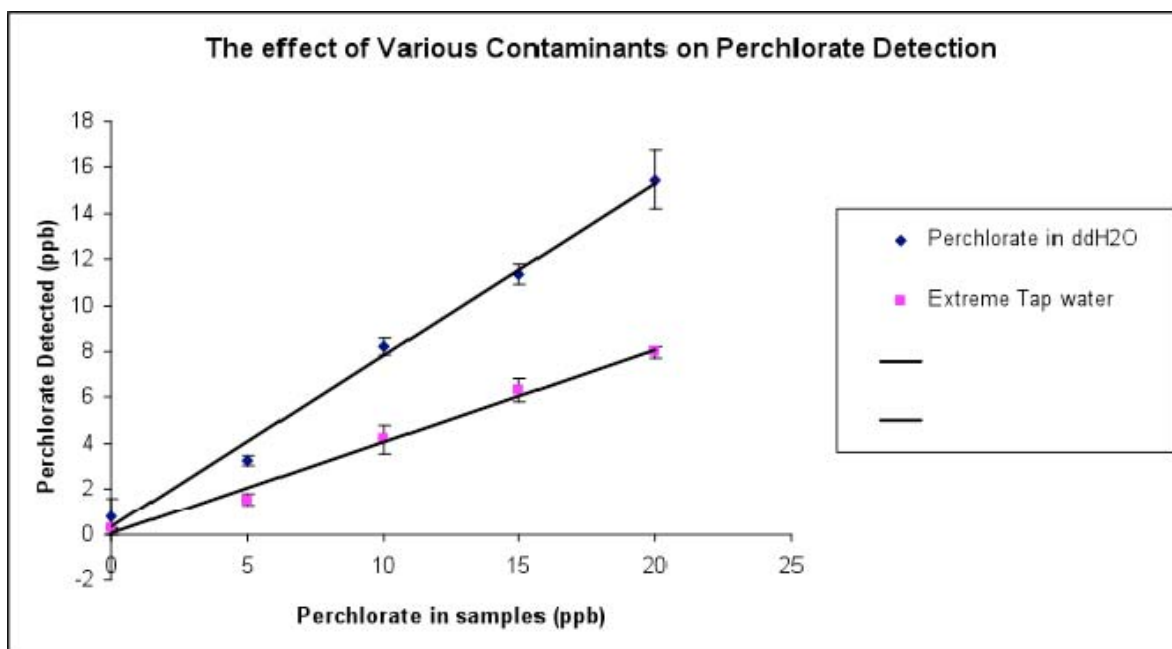
Figure 43. The effect of extreme tapwater on the SDVB columns: 200 mL samples of distilled water and extreme tapwater (ion concentration is shown above) were spiked with perchlorate. The spiked value is shown on the x-axis. After loading and eluting the samples off of the SDVB columns, perchlorate concentration were determined using ion chromatography. Perchlorate concentrations in the 2 mL samples were divided by 100 (dilution factor) to determine the original perchlorate concentration.

This interference would be significant enough to consistently underestimate the amount of perchlorate in tapwater, and most likely groundwater, samples. A similar observation was seen when other trialkylammonium molecules were used to bind perchlorate for a detection technique that used mass spectrometry (Magnuson et al., 2000). However, the amount of perchlorate that elutes has a strong linear correlation to the amount of perchlorate that is loaded on the column. In addition, the linear fits cross the y-intercept at approximately the origin, eliminating the possibility of false positives. Given these attributes, it was decided to analyze perchlorate

samples using the method of standard additions. In this technique, five samples are spiked with increasing concentrations of perchlorate. Ionic strength may have an effect on the binding of perchlorate to the SDVB columns, but it will also have a similar effect on the spiked perchlorate. This effect will allow the appropriate correlation of a signal in the bioassay to a concentration of perchlorate.

To test this technique, three samples collected from a perchlorate contaminated groundwater at the Aerojet facility, Sacremento CA were analyzed. The three samples were spiked with 0, 10, 20, 30, and 40 ppb perchlorate. As can be seen in figure 44, there is a linear correlation between the perchlorate spiked in the samples, and the signal intensity from wellhead sample 7069 (Aerojet estimated the concentration of perchlorate in this groundwater to be 6 ppb). Ion chromatography gave a perchlorate concentration of 9.6 ± 3.8ppb for this sample while the bioassay indicated a statistically similar value of 11.0 ± 1.0ppb. Similar results were seen for samples from other wellheads.
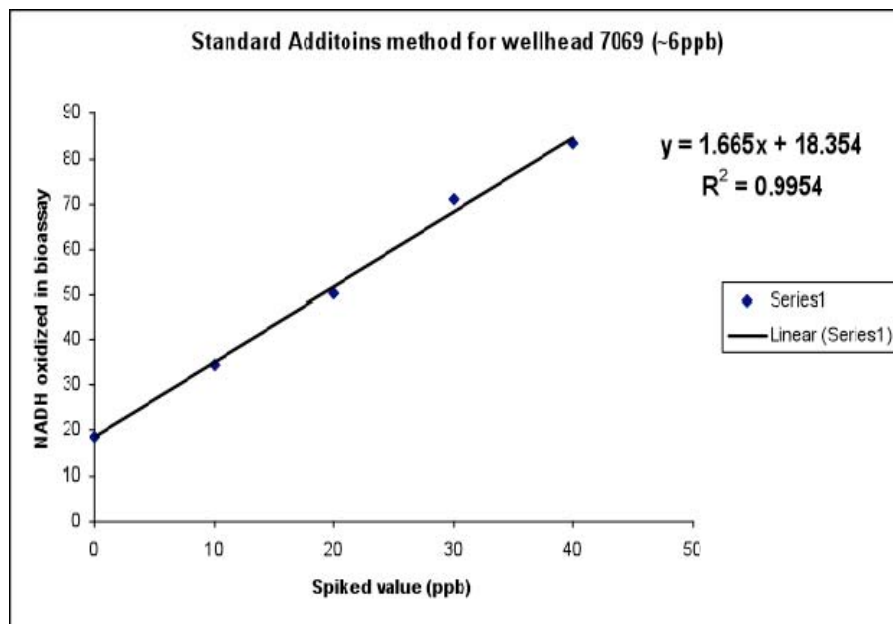
Figure 44 – Signal intensity vs. Spiked volume for a contaminated groundwater sample: The graph below shows spiked value on the x-axis vs. signal intensity. The x-intercept corresponds to the concentration of perchlorate in the groundwater sample.

The concentration of perchlorate in these samples is the absolute value of the x-intercept for the linear fits. The standard deviation is found using the equation:

$$St_{dev} = c_{unknown} \sqrt{((m_{error}/m)^2 + (b_{error}/b)^2)}$$

The variable ($c_{unknown}$) corresponds to the concentration of the unknown sample. The variables (m) and (m_{error}) correspond to the slope and the slope error of the linear fits, respectively. The variables (b) and (b_{error}) correspond to the y-intercept and the y-intercept error respectively. The slope error and y-intercept error were determined using the "Linest" function in Microsoft excel. The concentration of perchlorate in these samples is determined by the bioassay and compared to measurements made by ion chromatography in table 4 (ion chromatography measurements were made before concentration on the SDVB columns).

Table 4 – <u>Analysis of contaminated groundwater</u>: Concentrations of the wellheads were determined by analyzing the samples with ion chromatography and the bioassay standard addition method.

Sample	Determined by Ion Chromotograpy (ppb)	Determined by bioassay (ppb)
Wellhead (#7069)	9.6 +/- 3.8	11.0 +/- 1.0
Wellhead (#4590)	21.7 +/- 1.8	25.9 +/- 3.2
Wellhead (#4830)	70.5 +/- 5.4	88.3 +/- 16.3

Developed bioassay application

The efficacy of the bioassay was tested by analyzing tapwater and natural groundwater samples containing a range of perchlorate concentrations in the presence and absence of a range of potential interfering ions. The concentration of perchlorate determined by the bioassay was compared to measurements made by ion chromatography (table 1). Because of high concentrations, perchlorate in groundwater sample #4 was determined without the concentration step. Groundwater from this site was diluted 10 fold before analysis and perchlorate values were extrapolated from a standard curve. As shown in table 1, thirteen of the sixteen values determined by the anaerobic bioassay were within the standard deviations of the values determined by ion chromatography emphasizing its reliability and accuracy.

These results detail a reliable strategy for detecting perchlorate at low ppb range concentrations with a cheap, robust colorimetric assay. Because of perchlorate's stable and water-soluble nature, it remains a prevalent contaminant posing substantial health risk. Currently, its detection relies on ion chromatography, an expensive, time consuming procedure, requiring highly trained personnel. A new ion chromatography system can be as expensive as $50,000 US dollars, and has a consumables charge of ~$1 per sample. The assay developed in this case has a much lower instrument cost, merely a hand-held spectrophotometer and SDVB columns which are reusable. The consumables in this case could be as low as $0.13 per sample, although the method of standard additions increases this cost to $0.65. Nevertheless, the bioassay described here greatly decreases the cost of perchlorate detection through a decrease in the cost of both equipment and consumables.

Table 5 – Analysis of Tapwater samples: To make the 12 spiked tapwater samples, 10 mL samples were made with perchlorate in the ppm range. The samples were diluted 1000 fold in tapwater for bioassay analysis and analyzed directly by ion chromatography in triplicate (values shown assume a perfect 1000 fold dilution). To analyze each tapwater and envrironmental sample, five solutions of 200 mL were spiked with perchlorate in increments of 10 ppb from 0-40 ppb. Standard deviations for the bioassay were derived as indicated in the text.

Sample	Perchlorate determined by bioassay (ppb)	Perchlorate determined by IC (ppb)
Tapwater sample#1	0.77 +/- 1.7 ppb	0.15 +/- 0.01 ppb
Tapwater sample#2	2.2 +/- 1.9 ppb	1.65 +/- 0.22 ppb
Tapwater sample #3	0.8 +/- 2.4 ppb	2.36 +/- 0.15 ppb
Tapwater sample #4	4.0 +/- 3.2 ppb	3.35 +/- 0.06 ppb
Tapwater sample #5	2.6 +/- 2.5 ppb	4.30 +/- 0.06 ppb
Tapwater sample #6	2.2 +/- 2.8 ppb	6.28 +/- 0.39 ppb
Tapwater sample #7	5.3 +/- 1.2 ppb	6.65 +/- 0.11 ppb
Tapwater sample #8	13.8 +/- 4.5 ppb	7.88 +/- 0.17 ppb
Tapwater sample #9	9.0 +/- 2.9 ppb	13.45 +/- .04 ppb
Tapwater sample #10	20.5 +/- 1.7 ppb	16.51 +/- 4.51 ppb
Tapwater sample #11	18.8 +/- 1.9 ppb	21.58 +/- 4.80 ppb
Tapwater sample #12	31.6 +/- 7.8 ppb	25.68 +/- 2.50 ppb
Groundwater sample #1	11.0 +/- 1.0 ppb	9.6 +/- 3.8 ppb
Groundwater sample #2	25.9 +/- 3.2 ppb	21.7 +/- 1.8 ppb
Groundwater sample #3	88.3 +/- 16.3 ppb	70.5 +/- 5.4 ppb
Groundwater sample #4	19.74 +/- 2.83 ppm	16.73 +/- 5.46 ppm

If the legal perchlorate level in the United States is lowered nationwide, it is estimated that millions of people would be drinking tapwater that required additional purification (Renner, 2009). To identify perchlorate contaminated wells and reservoirs it is extremely important to have a quick reliable assay that can be done on-site to determine potential risks. As demonstrated here, this assay could readily be used to determine perchlorate in drinking waters nationwide for a fraction of current costs. As it is clear that ionic strength effects the detection of perchlorate by this assay, the lower limit of detection in groundwater is unclear. Before commercialization this assay needs to be validated with various perchlorate-contaminated groundwaters to ensure the viability of this assay as an environmental tool. However, data presented here indicate it is already a reliable tool for analyzing tapwater samples, as 75% of the 12 samples were found to have similar concentrations when analyzed by both the bioassay and the EPA approved ion chromatographic method (Hauntman et al., 1999). The average standard deviation for these samples is 2.8 ppb. This value could serve as a lower detection limit of the bioassay in tapwater.

Bioassay functional optimization (extraction protocol)
A method for the extraction and concentration of perchlorate from solution using bulk styrene divinyl benzene resin treated with decyltrimethylammonium (DTAB) in place of the syringe SPE columns was investigated for the purpose of optimizing the colorimetric bioassay. A total of fifteen variant methods were investigated during this period composed of four primary techniques with minor adjustments. All methods contained four basic steps; one, cleaning the resin or resin packets; two, incubating the resin with DTAB; three, loading the resin with perchlorate solution; and four, monitoring perchlorate concentration loss from the extracted liquid. Some variations of this procedure included using a coffee press, syringes pre-loaded with bulk resin beads, and closed jars on a shaker at different agitation speeds. Perchlorate concentrations in the extracted solutions were temporally monitored in all cases by ion chromatography with conductivity detection as a measure of perchlorate extraction effectiveness. Of the methods tested, the best extraction of perchlorate resulted from the placement of packets (teabags) each containing 1 gram of resin in beakers containing 200ml aqueous perchlorate solutions (200μM) on a magnetic stirrer (Fig. 45). Use of this method resulted in extraction of

more than 75% percent of perchlorate from the solution within a 15-minute time frame (Fig. 46) which is comparable to that achievable using commercial SPE syringe columns but without the need of forcing large samples through flow resistant column filters. A minor increase in perchlorate concentration was observed in the control, which indicates a margin of error in either the analysis of the samples, or within the procedure, or both (data not show). However, the results of the tests samples are encouraging and represent a good path forward for enhancing the bioassay logistical protocol. This refined process in combination with the continuing steps of the bioassay, can lower the cost, time, equipment, and specialized personnel needed to identify

Figure 45. Extraction of perchlorate from 200ml samples using 1g of DTAB treated SDVB resin in teabags.

concentrations of perchlorate in environmental water samples.
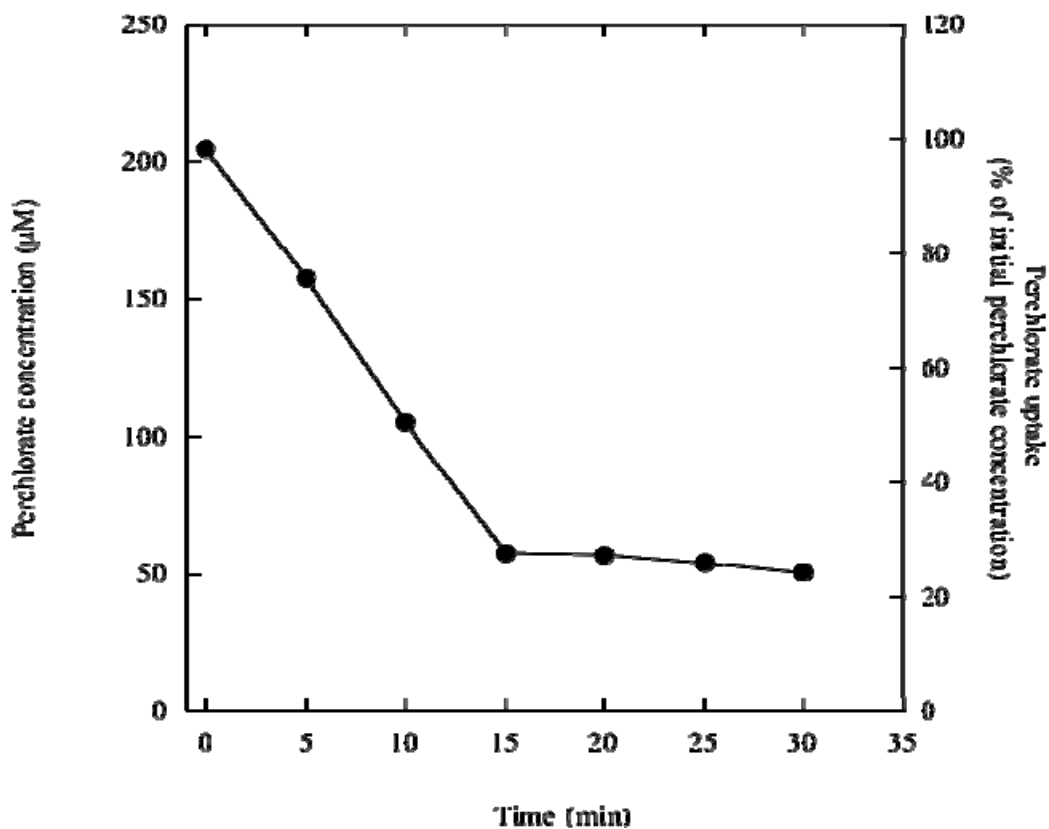
Figure 46. Perchlorate uptake by bulk resin encased in a submerged teabag in 200ml of a perchlorate-contaminated sample.

Clone expression of function perchlorate reductase in E. coli.

To aid in the mass production of the perchlorate reductase for bioassay commercialization, we investgated the overexpression of the functional enzyme in *E. coli* using a polyhistidine tag to simplify subsequent protein purification. We had previously determined that the perchlorate reductase enzyme is composed of two structural subunits α and β encoded by the *pcrA* and *pcrB* gene, respectively. The *pcrAB* region of the perchlorate reductase operon was PCR-amplified using primers pcrA-For (5'-CACCATGGTTCAAATGACACGAAGA-3') and pcrB-Rev (5'-GGTCAAAGGAGAAATCATCAT-3'). The 3.8 kb PCR product was then inserted into the *E. coli* expression vector pBAD202/D-TOPO (Invitrogen, CA) using vector-specific overhangs for unidirectional orientation of the insert according to the product manual. The correct orientation of the insert was confirmed by additional PCR using a vector-specific forward primer (Txn-For, 5'-ttcctcgacgctaacctg-3') and the pcrB-Rev primer in one reaction and a vector-specific reverse

primer (pBAD-Rev, 5'-gatttaatctgtatcagg-3') and the pcrA-For primer in another reaction. In addition, single digests of the recombinant vector using restriction enzyme SmaI or SacI were performed to verify insert size.
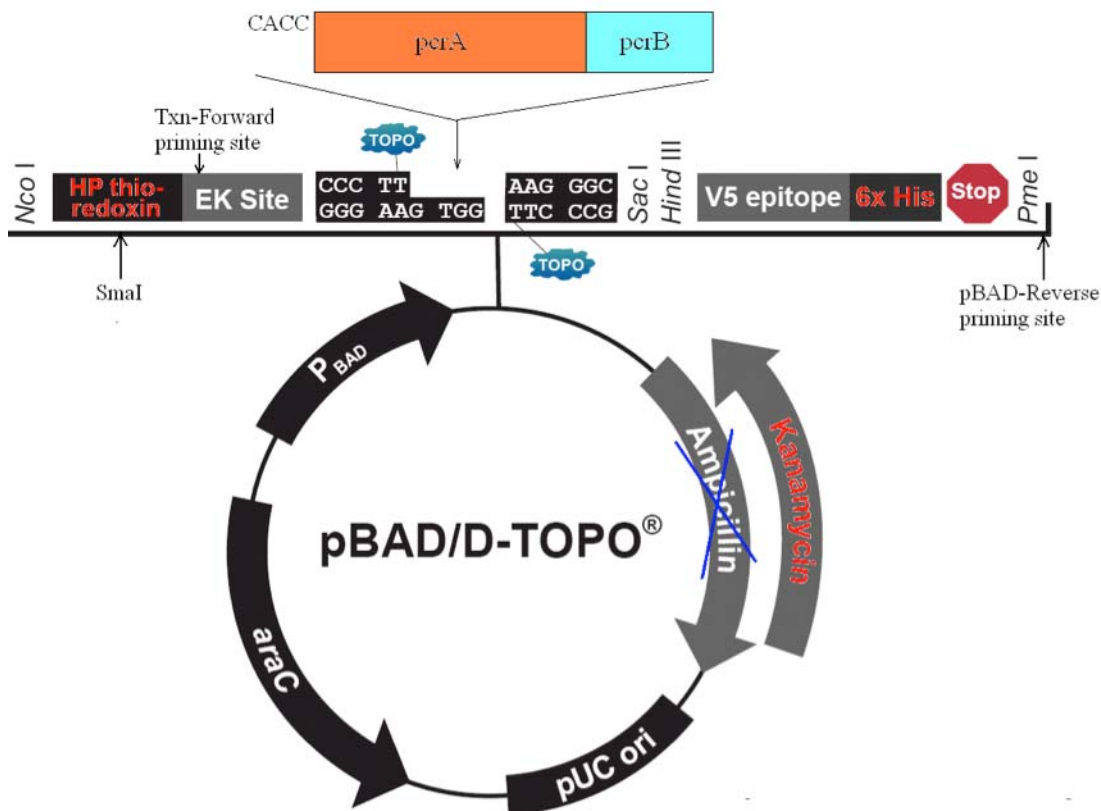
Figure 47: Visual model of the pBAD-*pcrAB* expression vector

The original linearized vector contained an *araBAD* promoter and fusion protein-encoding gene on one end and a histidine (His) tag-encoding region on the other end. Thus, once the *pcrAB* PCR product was inserted, the orientation of the *pcrAB* genes was such that the translation products include the PcrA polypeptide with the fusion protein fused to the N-terminus and the PcrB polypeptide with the His tag fused to the C-terminus (Fig. 47). This vector puts the inserted genes under the control of the *araBAD* promoter such that gene expression is switched on in the presence of L-arabinose; thus, protein levels can be optimized to ensure maximum expression. The N-terminal His-Patch thioredoxin is for increased translation efficiency and solubility of heterologous proteins. When overexpressed in *E. coli*, thioredoxin is able to accumulate to approximately 40% of the total cellular protein and still remain soluble. When

used as a fusion partner, thioredoxin can increase translation efficiency and solubility of proteins expressed in *E. coli*. In addition, the metal-binding domain encoded by a polyhistidine tag carried on the vector allows for rapid purification of the recombinant protein by immobilized metal affinity chromatography. A vector-encoded kanamycin resistance cassette allows for direct selection of transformants (Fig. 47).

The recombinant pBAD202-*pcrAB* expression plasmid was transformed into chemically competent *E. coli* TOP10 cells according to standard transformation procedures and the resulting transformant pool was plated onto LB-kanamycin (LB-Kan) plates for overnight incubation at 37˚C. A single colony was selected from which the plasmid was purified and tested using both the PCR amplifications and single restriction digests described above to confirm insert size and orientation. *In vitro* protein expression was then performed according to the manufacturer's instructions. Briefly, pBAD202-*pcrAB* -containing *E. coli* cells were cultured at 37˚C to mid-log phase (OD = ~0.5) at which time 0.2% arabinose was added to induce expression of the cloned *pcrAB* genes. The culture was incubated for another 4 hours and the cells were harvested. Successful expression of the target protein was demonstrated by denaturing SDS-page analysis. Native PcrA and PcrB protein subunits were included as size controls on the SDS-page gel. When compared to a plasmid-less *E. coli* negative control, the expression of the PcrA polypeptide subunit was clearly observed on the SDS-PAGE gel. (Fig. 48, lane 4 and lane 6. Note that the cloned PcrA subunit is larger than the native subunit because of the fusion protein attached to the N-terminus.) However, although the PcrB subunit was expressed when cloned by itself (Fig. 48, lane 5), the expression of the PcrB polypeptide subunit from the pBAD202-*pcrAB* plasmid was much lower in comparison (Fig. 48, lane 6), possibly due to differential translation of the *pcrB* gene which was located further downstream of the pBAD promoter than the *pcrA* gene and thus resulted in a lower expression rate.
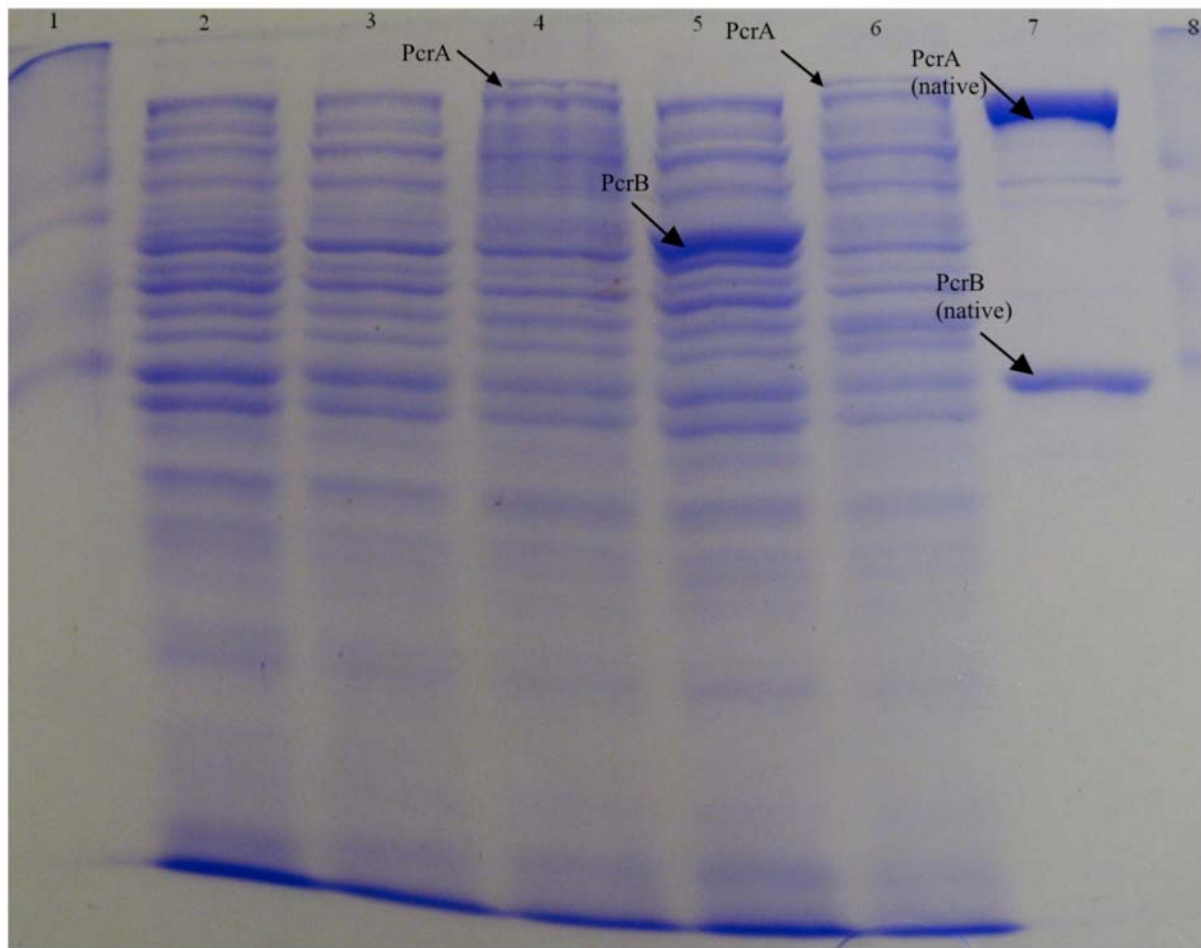
Figure 48: SDS-PAGE gel of perchlorate reductase α and β subunits expressed from pBAD-*pcrAB*, pBAD-*pcrA*, and pBAD-*pcrB* expression vectors. Lane 1) Protein size marker; lane 2) *E. coli* negative control (no plasmid); lane 3) pBAD-*pcrAB* expression vector, uninduced; lane 4) pBAD-*pcrA* expression vector; lane 5) pBAD-*pcrB* expression vector; lane 6) pBAD-*pcrAB* expression vector; lane 7) native purified perchlorate reductase; lane 8) protein size marker. Note that the recombinant PcrA and PcrB subunits are larger than the native subunits because of the addition of the thioredoxin fusion protein to PcrA and the polyhistidine tag to PcrB.

The Ni-NTA Purification System is designed for purification of 6xHis-tagged recombinant proteins expressed in bacteria, insect and mammalian cells. The system is designed around the high affinity and selectivity of Ni-NTA agarose for recombinant fusion proteins that are tagged with six histidine residues. The purification procedure was performed according to the manufacturer's instructions. Briefly, *E. coli* cells was lysed by guanidine hydrochloride lysis buffer containing up to 0.2% sarkosyl to ensure protein solubility, centrifuged briefly to pellet cell debris, and the supernatant loaded onto a Ni-NTA column. Upon passing through the column, the histidine residues on the His-tagged protein bound to the nickel in the column matrix. The column was then washed with denaturing buffer at pH 6.0 followed by a second wash at pH 5.3 to remove all non-specific (non-His tagged) proteins from the column matrix. Finally, the His-tagged protein was eluted from the column by washing with elution buffer at pH 4.0. When tested the resultant protein was functionally inactive (data not shown). Inactivity may have been the result of protein denaturation during purification or the production of an inactive protein by the *E. coli* transformant. As our subsequent studies with cell lysates of *D. agitata* indicated the low cost and easy production of large quantities of functional protein that was readily applicable to the bioassay, work on the expression of the perchlorate reductase in *E. coli* ceased.

Conclusions

A highly sensitive, robust, and inexpensive benchtop colorimetric bioassay was developed for the determination of parts per billion (ppb or $\mu g.L^{-1}$) perchlorate. In its current form the bioassay uses the partially purified perchlorate reductase (PCR) enzyme from *Dechloromonas agitata* to detect perchlorate with the redox active dye phenazine methosulfate and nicotine adenine dinucleotide. By using a specific addition scheme and covering all reactions with mineral oil, the reaction could be performed on the benchtop, with a lower detection limit of 200 ppb. When combined with perchlorate purification and concentration by solid phase extraction (SPE) the detection limit was reduced to 2 ppb. Perchlorate was eluted from the SPE column using a solution of 2 M NaCl and 200 mM morpholine propane sulfonic acid (pH 12.5). By applying this assay with the method of standard additions, the efficacy of the bioassay was demonstrated by analyzing perchlorate samples (2 – 17,000 ppb) in tapwater and contaminated groundwater. This report describes the development of the simple robust bioassay for the detection of

perchlorate which is an important emerging contaminant that poses a significant global health threat. Perchlorate is known to affect thyroid function in mammals and its toxicity primarily results from its inhibition of thyroid hormone output. Perchlorate binds to the sodium-iodide symporter and consequently competitively inhibits iodide uptake by the thyroid gland. Thyroid hormones are synthesized from iodide in the thyroid and are responsible for regulating mammalian metabolism. Long term reduction in iodide uptake in an adult can ultimately result in hypothyroidism. Furthermore, because the thyroid hormones are required for normal physical and mental development, exposure to thyroid inhibitors such as perchlorate may have a direct impact on fetal and infant neuropsychological development. Previous studies have indicated that children of mothers suffering from maternal thyroid deficiency during pregnancy performed below average on 15 tests relating to intelligence, attention, language, reading ability, school performance and visual-motor performance.

Before 1997, perchlorate was an unregulated compound in the US. However, the discovery of perchlorate contamination in drinking water resources throughout the US especially those in the southwestern states of Nevada, Utah, and California prompted the establishment of a provisional action level of 18 μg.L^{-1} in 1997. The worst case was discovered in the Las Vegas, Nevada area where perchlorate has been manufactured for more than fifty years and groundwater contamination was discovered ranging from 630,000 μg.L^{-1} to 3,700,000 μg.L^{-1}. In 1998 perchlorate was added to the US EPA Contaminant Candidate List for drinking water supplies and in January 2002, as a result of the publication of a US EPA draft review on toxicological and risk assessment data associated with perchlorate contamination, a revised and lowered health protective standard of 1 μg.L^{-1} was suggested which resulted in a decade of high profile debate over the determination of a final federal action level. Most US states have subsequently adopted their own regulatory recommended limit with values in the order of 6 μg.L^{-1}.

Perchlorate is principally a synthetic compound and its salts have a broad range of different industrial applications ranging from pyrotechnics to lubricating oils. Its presence in the environment predominantly results from legal historical discharge of unregulated manufacturing waste streams, leaching from disposal ponds, and from the periodic servicing of military inventories. To date, the only significant natural source of perchlorate known is associated with

mineral deposits found in Chile where the perchlorate content averages as much as 0.03% of the total mineral mass. Throughout the last century, the Chilean ore deposits were extensively mined as a mineral and nitrate source for fertilizer manufacture, and the perchlorate often persisted throughout processing into the final product at low concentrations.

Furthermore, the presence of perchlorate has been indicated in a variety of other natural phosphorous-bearing minerals formed through evaporation processes (evaporites) collected from a diversity of arid locations. More recently, it was demonstrated that solid fertilizers not derived from the Chilean deposits and commonly used for the hydroponic growth of various fruit and vegetables can contain perchlorate at concentrations as high as 350 $\mu g.kg^{-1}$. Such levels could represent a significant global health threat owing to the increasing use of hydroponic farming techniques for the production of a wide variety of plants for human consumption throughout the world. Studies performed on different plant species grown in soils containing perchlorate have indicated uptake and in certain plant species such as tobacco and lettuce the perchlorate accumulates and persists during processing into the final shelf products, such as cigarettes, cigars and chewing tobacco, at concentrations as high as 60 $mg.kg^{-1}$.

These facts combined with the findings of several other studies, underscored by its recent discovery in Martian soils, have indicated that the true extent of perchlorate contamination and its natural abundance have been severally underestimated. The most common analytical method currently available is an ionic chromatographic technique with conductivity detection that was developed in the mid 1990's which forms the basis of the current EPA Method 314 for the determination of perchlorate in drinking water. Although a dependable technique, perchlorate identification is based on elution times in comparison to standards rather than specific molecular structure. This type of determination allows for a significant margin of error and interference. Several more sensitive and accurate techniques including complexation electrospray mass spectrometry, tandem electrospray mass spectrometry, high-field asymmetric waveform ion mobility spectrometry, and Raman spectrometry have been developed and applied to a broad range of environmental samples. Although these techniques have proved to be accurate for identification of perchlorate concentrations in the sub-$\mu g.L^{-1}$ range in many complex matrices

they are laborious, expensive, time consuming, and require highly trained personnel making them unsuitable for the rapid delineation of contaminated environments.

The work outlined in this report resulted in the development of an alternative biochemical technique with a detection limit of 2 μg.L^{-1}, which is below the recommended regulatory limit adopted by most US states. This bioassay uses partially purified enzymes from the perchlorate reducing bacterium *Dechloromonas agitata* strain CKB in a colorimetric reaction. The strain CKB perchlorate reductase enzyme (PCR) quantitatively reduces perchlorate to chlorite, while oxidizing the biological cofactor NADH. NADH is widely used in biological reactions because of its stability and well-characterized molar extinction coefficient. This technique provides a rapid, specific, and sensitive method for the detection of perchlorate that obviates the need for expensive equipment and highly trained personnel. A preliminary patent submission has been made to the US Patent Office on this bioassay.

Summary of Assay

1. Total assay volume is 1 ml.

2. Collected aqueous samples (1 L) are separated into 5x200ml aliquots and spiked with 0, 10, 20, 30, and 40 ppb perchlorate respectively.

3. The spiked samples are prepared for bioassay analysis by SPE extraction using 1ml SDVB columns treated with DTAB.

4. The loaded columns are washed with 5 mls of 2.5 mM DTAB and 15% acetone to elute competing ions (nitrate/chlorate).

5. The perchlorate is eluted from the columns with 2mls of 2 M NaCl and 200 mM MOPS (pH 13).

6. The pH of the perchlorate containing eluent is neutralized using 1 M HCl (final pH 7.5, checked by pH paper).

7. 1 ml of this pH adjusted eluent is used for the bioassay and is added to a 3 ml glass cuvette.

8. The solution is covered with 1 ml mineral oil.

9. PMS and NADH are added underneath the mineral in final concentrations of 175 μM and 1 mM respectively.

10. Once the yellow color disappears (approx. 5 mins), the initial absorbance (I_{abs}) at 340 nm is taken (this is equivalent to the starting NADH concentration).

11. PCR is added to the solution underneath the mineral oil to a final concentration of 48 μg/ml.

12. Samples are incubated at 40 °C.

13. The final absorbances (F_{abs}) are taken after 20 minutes.

14. The difference $I_{abs} - F_{abs} = \Delta_{abs}$ represents the loss of NADH coupled to perchlorate reduction for each sample.

15. The perchlorate concentration is calculated from a plot of Δ_{abs} vs. spiked perchlorate concentration for each contaminated groundwater sample: The x-intercept of the graph corresponds to the concentration of perchlorate in the groundwater sample.

References.

Achenbach, L. A., K. S. Bender, Y. Sun and J. D. Coates. (2006. The biochemistry and genetics of microbial perchlorate reduction. *In* B. Gu and J. D. Coates. *Perchlorate, environmental occurrence, interactions, and treatment.* . Springer Publishers, MA.

Aslander, A. 1928. Experiments on the eradication of canada thistle, cirsium arvense, with chlorates and other herbicides. *J. Agric. Res.* **36**: 915-915.

Bender, K. S., M. R. Rice, W. H. Fugate, J. D. Coates and L. A. Achenbach. 2004. Metabolic primers for the detection of (per)chlorate-reducing bacteria in the environment. *Appl Environ Microbiol.*

Brown, G. M. and B. Gu. (2006. The chemistry of perchlorate in the environment, 24. *In* B. G. a. J. Coates. *Perchlorate: Environmental occurance, interactions, and treatment.* Springer. New York.

Bruce, R. A., L. A. Achenbach and J. D. Coates. 1999. Reduction of (per)chlorate y a novel organism isolated from a paper mill waste. . *Environ Microbiol* **1**: 319-331.

Bryan, E. H. 1964). Combined oxygen sources for measurement of the strength of wastes. Annual meeting of the North Carolina section, American Water Works Association and the North Carolina Water Pollution Control Association, Raleigh, NC.

Bryan, E. H. 1966. Application of the chlorate bod procedure to routine measurement of wastewater strength. *J. Wat. Pollut. Cont. Fed.* **38**: 1350-1362.

Bryan, E. H. and G. A. Rohlich. 1954. Biological reduction of sodium chlorate as applied to measurement of sewage bod. *Sewage Ind. Waste* **26**: 1315-1324.

Chaudhuri, S. K., S. M. O'Connor, R. L. Gustavson, L. A. Achenbach and J. D. Coates. 2002. Environmental factors that control microbial perchlorate reduction. *Appl Environ Microbiol* **68**: 4425-4430.

Christen, K. 2003. Epa perchlorate decision takes many by surprise. *Environmental Science & Technology* **37**: 347A-348A.

Clark, J. J. J. (2000. Toxicology of perchlorate. *In* E. T. Urbansky. *Perchlorate in the environment.* Kluwer Academic/Plenum. New York.

Coates, J. D. and L. A. Achenbach. 2004. Microbial perchlorate reduction: Rocket-fuelled metabolism. *Nature Rev. Microbiol.* **2**: 569-580.

Coates, J. D. and A. Jackson. (2008. Principles of perchlorate treatment. *In* C. H. Ward and H. F. Stroo. *In situ bioremediation of perchlorate in groundwater.* Springer. Norwell, MA.

Coates, J. D., U. Michaelidou, R. A. Bruce, S. M. O'Connor, J. N. Crespi and L. A. Achenbach. 1999. Ubiquity and diversity of dissimilatory (per)chlorate-reducing bacteria. *Applied and Environmental Microbiology* **65**: 5234-5241.

Coates, J. D., U. Michaelidou, S. M. O'Connor, R. A. Bruce and L. A. Achenbach. (2000. The diverse microbiology of (per)chlorate reduction., 257-270. *In* E. D. Urbansky. *Perchlorate in the environment.* Kluwer Academic/ Plenum. New York.

Collette, T. W., T. L. Williams, E. T. Urbansky, M. L. Magnuson, G. N. Hebert and S. H. Strauss. 2003. Analysis of hydroponic fertilizer matrixes for perchlorate: Comparison of analytical techniques. *Analyst* **128**: 88-97.

Danielsson-Thorell, H., K. Stenklo, J. Karlsson and T. Nilsson. 2003. A gene cluster for chlorate metabolism in ideonella dechloratans. *Appl. Environ. Microbiol.* **69**: 5585-5592.

de Groot, G. N. and A. H. Stouthamer. 1969. Regulation of reductase formation in proteus mirabilis. I. Formation of reductases and enzymes of the formic hydrogenlyase complex in the wild type and in chlorate resistant mutants. *Arch. Microbiol.* **66**: 220-233.

Ellington, J. J., N. L. Wolfe, A. W. Garrison, J. J. Evans, J. K. Avants and Q. C. Teng. 2001. Determination of perchlorate in tobacco plants and tobacco products. *Environmental Science & Technology* **35**: 3213-3218.

Ericksen, G. E. 1983. The chilean nitrate deposits. *American Scientist* **71**: 366-374.

Fan, A. M., R. Howd, D. Ting and G. V. Allexeeff (2004). Public health goal for perchlorate in drinking water, California Environmental Protection Agency.

Fultz, M. L. and R. A. Durst. 1982. Mediator compounds for the electrochemical study of biological redox systems - a compilation. *Analytica Chimica Acta* **140**: 1-18.

Hackenthal, E. 1965. Die reduktion von perchlorat durch bacterien. Ii. Die identitat der nitratreduktase und des perchlorat reduzierenden enzyms aus b. Cereus. *Biochem. Pharm.* **14**: 1313-1324.

Hackenthal, E., W. Mannheim, R. Hackenthal and R. Becher. 1964. Die reduktion von perchlorat durch bakterien. I. Untersucungen an intaken zellen. *Biochem. Pharmacol.* **13**: 195-206.

Haddow, J. E., G. E. .Palomaki, W. C. Allan, J. R. Williams, G. J. Knight, J. Gagnon, C. E. O'Heir, M. L. Mitchell, R. J. Hermos, S. E. Waisbren, J. D. Faix and R. Z. Klein. 1999. Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. *New Eng J Med* **341**: 549-555.

Hauntman, D. P., D. J. Munch, A. D. Eaton and A. W. Haghani (1999). Determination of perchlorate in drinking water using ion chromotography, revision 1 method 314, doc no epa/815/b-99/003. U. E. P. Agency.

Heider, J. and A. Bock. 1993. Selenium metabolism in micro-organisms. *Adv. MIcrob. Physiol.* **35**: 71-109.

Heinnickel, M., G. Shen, R. Agalarov and J. H. Golbeck. 2005. Resolution and reconstitution of a bound fe-s protein from the photosynthetic reaction center of heliobacterium modesticaldum. *Biochemistry* **44**: 9950-9960.

Held, P. (2006). Determination of NADH concentrations with the synergy 2 multi-detection microplate reader using fluorescence or absorbance. Winooski, VT, Biotek Instruments.

Hogue, C. 2003. Rocket-fueled river. *Chemical & Engineering News* **81**: 37-46.

Kengen, S. W., G. B. Rikken, W. R. Hagen, C. G. van Ginkel and A. J. Stams. 1999. Purification and characterization of (per)chlorate reductase from the chlorate-respiring strain gr-1. *J Bacteriol* **181**: 6706-6711.

Kirk, A. B., E. E. Smith, K. Tian, T. A. Anderson and P. K. Dasgupta. 2003. Perchlorate in milk. *Environmental Science & Technology* **37**: 4979-4981.

Little, P. A. and Y. Subbarow. 1945. Use of refined serum albumin as a nutrient for t-pallidum. *Journal of Immunology* **50**: 213-219.

Magnuson, M. L., E. T. Urbansky and C. A. Kelty. 2000. Determination of perchlorate at trace levels in drinking water by ion-pair extraction with electrospray ionization mass spectrometry. *Anal Chem* **72**: 25-29.

Mathews, C. K., K. E. van Holde and K. Ahern. 1999. *Biochemistry*. New York, NY, Prentice Hall.

Michaelidou, U., L. A. Achenbach and J. D. Coates. (2000. Isolation and characterization of two novel (per)chlorate -reducing bacteria from swine waste lagoons., 271 - 283. *In* E. D. Urbansky. *Perchlorate in the environment*. Kluwer Academic/ Plenum. New York.

Motzer, W. E. 2001. Perchlorate: Problems, detection, and solutions. *Environmental Forensics* **2**: 301-311.

Neidhardt, F. C., R. Curtiss, J. Ingraham, E. Lin, K. Brooks Low, B. Magasanik, W. Rfznikopp, M. Riley, M. Schaechter and H. E. Umbarger, Eds. 1996. <u>Escherichia coli and salmonella - cellular and molecular biology</u>. Washington DC, ASM Press.

O'Connor, S. M. and J. D. Coates. 2010. A novel enzymatic assay for low level chlorite determination. *J Anal Chem* (**submitted**).

Okeke, B. C., T. Giblin and W. T. Frankenberger. 2002. Reduction of perchlorate and nitrate by salt tolerant bacteria. *Environmental Pollution* **118**: 357-363.

Orris, G. J., G. J. Harvey, D. T. Tsui and J. E. Eldrige (2003). Preliminary analyses for perchlorate in selected natural materials and their derivative products. Open-file 03-314. <u>US Geological Survey</u>.

Rao, B., T. A. Anderson, G. J. Orris, K. A. Rainwater, S. Rajagopalan, R. M. Sandvig, B. R. Scanlon, D. A. Stonestrom, M. A. Walvoord and W. A. Jackson. 2007. Widespread natural perchlorate in unsaturated zones of the southwest united states. *Environ Sci Technol.* **41**: 4522-4528.

Renner, R. 1998. Perchlorate-tainted wells spur government action. *Environ. Sci. Technol.* **32**: 210A-210A.

Renner, R. 1999. Epa draft almost doubles safe dose of perchlorate in water. *Environmental Science & Technology* **33**: 110a-111a.

Renner, R. 2003. Perchlorate regulation faces further delay. *Environmental Science & Technology* **37**: 166a-167a.

Renner, R. 2009. Epa perchlorate decision flawed, say advisers. *Environmental Science & Technology* **43**: 553-554.

Rikken, G., A. Kroon and C. van Ginkel. 1996. Transformation of (per)chlorate into chloride by a newly isolated bacterium: Reduction and dismutation. *Appl. Microbiol. Biotechnol.* **45**: 420-426.

Roldan, M. D., F. Reyes, C. Moreno-Vivian and F. Castillo. 1994. Chlorate and nitrate reduction in the phototrophic bacteria rhodobacter capsulatus and rhodobacter sphaeroides. *Curr. Microbiol.* **29**: 241-245.

Roote, D. S. (2001). Technology status report perchlorate treatment technologies first edition. Pittsburgh, Ground-Water Remediation Technologies Analysis Center**:** 1-40.

Schilt, A. A. 1979. *Perchloric acid and perchlorates.* Ohio, The G. Fredrick Smith Chemical Comany.

Stanbury, J. B. and J. B. Wyngaarden. 1952. Effect of perchlorate on the human thyroid gland. *Metabolism-Clinical and Experimental* **1**: 533-539.

Stenklo, K., H. D. Thorell, H. Bergius, R. Aasa and T. Nilsson. 2001. Chlorite dismutase from ideonella dechloratans. *J. Biol. Inorgan. Chem.* **6**: 601-607.

Stewart, V. 1988. Nitrate respiration in relation to facultative metabolism in enterobacteria. *Microbiol. Rev.* **52**: 190-232.

Stumm, W. and J. J. Morgan. 1996. *Aquatic chemistry: Chemical equilibria and rates in natural waters.* New York, NY, John Wiley & Sons , Inc.

Susarla, S., S. T. Bacchus, G. Harvey and S. C. McCutcheon. 2000. Phytotransformations of perchlorate contaminated waters. *Environmental Technology* **21**: 1055-1065.

Thorne, P. G. (2004). Field screening method for perchlorate in water and soil. South Royalton, Vermont, ERDC/CRREL.

Urbanski, T. 1988. Composite propellants, 602-620. *In. Chemistry and technology of explosives.* Pergamon Press. Oxford, **4**.

Urbansky, E. T. 1998. Perchlorate chemistry: Implications for analysis and remediation. *Bioremediation Journal* **2**: 81-95.

Urbansky, E. T. 2002. Perchlorate as an environmental contaminant. *Environ Sci Pollut Res Int* **9**: 187-192.

Urbansky, E. T. and S. K. Brown. 2003. Perchlorate retention and mobility in soils. *Journal of Environmental Monitoring* **5**: 455-462.

Urbansky, E. T., S. K. Brown, M. L. Magnusson and C. A. Kelty. 2001. Perchlorate levels in samples of sodium nitrate fertilizer derived from chilean caliche. *Environ. Pollut.* **112**: 299-302.

USEPA (1998). Drinking water candidate contaminanat list. Washington DC, US Environmental Protection Agency.

Van Aken, B. and J. L. Schnoor. 2002. Evidence of perchlorate (cio4-) reduction in plant tissues (poplar tree) using radio-labeled (cio4-)-c-36. *Environmental Science & Technology* **36**: 2783-2788.

van Ginkel, C., G. Rikken, A. Kroon and S. Kengen. 1996. Purification and characterization of chlorite dismutase: A novel oxygen-generating enzyme. *Arch. Microbiol.* **166**: 321-326.

Waldman, P. (2003). Pentagon hid pollution report lawmakers say. <u>Wall Street Journal</u>. New York.

Wallace, W., T. Ward, A. Breen and H. Attaway. 1996. Identification of an anaerobic bacterium which reduces perchlorate and chlorate as wolinella succinogenes. *J. Ind. Microbiol.* **16**: 68-72.

Wirt, K., J. Laikhlman, J. Rohrer and P. Jackson. 1998. Low-level perchlorate analysis in drinking water and groundwater by ion chromotography. *Am Environ Lab* **10**: 1-5.

Wolff, J. 1998. Perchlorate and the thyroid gland. *Pharmacological Reviews* **50**: 89-105.

Appendix A

Publications Arising from this Project

Articles in Peer Reviewed Journals

1. Heinnickel, M., Smith, S., Koo, J., Chan, E., O'Connor, S.M., and Coates, J.D. (2010). Design and optimization of a colorimetric bioassay for perchlorate with a lower detection limit of 2 ppb. Environ. Sci. Technol. (submitted)

2. Thrash, J.C., Baker, B.J., Ahmadi, S., Torok, T., and Coates, J.D. (2010) *Magnetospirillum bellicus* sp. nov., a novel dissimilatory perchlorate-reducing bacterium in the *Alphaproteobacteria* isolated from a bioelectrical reactor. Appl. Environ Microbiol. 76:4730-4737

3. Thrash, J.C., Pollock, J., Torok, T., and Coates, J.D. (2009). Novel dissimilatory perchlorate-reducing bacteria in the *Betaproteobacteria* outside of the *Dechloromonas* and *Azospira*, and description of *Dechlorobacter hydrogenophilus* gen. nov., sp. nov., and *Propionivibrio militaris*, sp. nov. Appl. Microbiol. Biotechnol. 86:335–343

4. Sun, Y., Ali, N., Gustavson, R.L., Weber, K.A., Coates, J.D. (2009). Taxis of dissimilatory perchlorate-reducing bacteria to various chemical attractants. Appl. Microbiol Biotechnol. Online First Jun 17th, 2009; 84: 955–963

5. Van Trump, J.I. and Coates, J.D. (2008). Thermodynamic targeting of microbial perchlorate reduction by selective electron donors. ISME J. (Dec 18th 2008 Advance publication). (2009) 3:466–476

Book Chapters

1. Kaser, F. and Coates, J.D. (2009). Nitrate, Perchlorate, and Metal Respirers. In *Handbook of Hydrocarbon and Lipid Microbiology*. Timmis, K.N. (ed) Springer-Verlag Berlin Heidelberg pp. 2034-2047

2. Coates, J. D. and A. Jackson (2008). Principles of perchlorate treatment. In Situ Bioremediation of Perchlorate in Groundwater. C. H. Ward and H. F. Stroo. Norwell, MA, Springer.

3. Coates, J.D. and Achenbach, L.A. (2006). The microbiology of perchlorate reduction and its bioremediative application. *In* Gu, B. and Coates, J.D. (Eds) *Perchlorate, Environmental Occurrence, Interactions, and Treatment*. Springer Publishers, MA

4. Achenbach, L.A., Bender, K.S., Sun, Y., and Coates, J.D. (2006). The biochemistry and genetics of microbial perchlorate reduction. *In* Gu, B. and Coates, J.D. (Eds) *Perchlorate, Environmental Occurrence, Interactions, and Treatment*. Springer Publishers, MA

Books

1. Gu, B. and Coates, J.D. (Editors) (2006) Perchlorate, Environmental Occurrence, Interactions, and Treatment. Springer Publishers, MA

Patent Applications

1. A colorimetric bioassay for perchlorate (2007) John D. Coates and Mark Heinnickel

Conference/Symposium Abstracts

1   Coates, J.D. (2009). Microbial perchlorate reduction – a rocket fueled metabolism. The New Martian Chemistry Workshop July 27-28, Medford, MA.

2   Thrash, J.C., Torok, T, and Coates, J.D. (2009). "A novel isolate from a new dissimilatory perchlorate-reducing clade in the *Betaproteobacteria*." American Society for Microbiology 109th General Meeting.

3   Coates, J.D. (2009). "Microbial perchlorate reduction – a rocket fueled metabolism". International Water Association/Groundwater Resources Association of California Micropol & Ecohazard 2009.

4   Sun, Y. and Coates, J.D. (2008) "Nitrate or Perchlorate: Preferential Utilization of Electron Acceptors in *Dechloromonas aromatica* strain RCB". 108th American Society for Microbiology General Meeting. June 1 – 5. Boston, MA

5   Thrash, J.C. and Coates, J.D. (2009). "Continuous Bioelectrical Perchlorate Remediation." International Water Association/Groundwater Resources Association of California Micropol & Ecohazard 2009.

6   Coates, J.D., Smith, S.C., Chan, E., Chow, C., DuBois, J., Igarashi, R.Y., Hernandez, J.A. (2006). A Highly Sensitive Colorimetric Bioassay for Perchlorate. In: Abstracts of the Partners in Environmental Technology Symposium and Workshop, Washington DC.

7   Byrne-Bailey, K. G., Kaser, F.M. and Coates, J. D. (2008) The Genome of *Dechloromonas aromatica* strain RCB and Redundancy in the Genetic Pathway for Aerobic Hydrocarbon Oxidation. JGI Users Meeting, Walnut Creek, CA.

8   Sun, Y., K. A. Weber, N. Ali, J. D. Coates. (2006) Regulation of Microbial Perchlorate Reduction by Perchlorate. 106th General Meeting American Society for Microbiology, Orlando, FL. May 21-25

9   Thrash, J.C., Achenbach, Coates, J.D. (2008). "*Dechlorospirillum* VDY: a novel dissimilatory perchlorate-reducing bacteria capable of mesophilic anaerobic iron oxidation." International Society for Microbial Ecology 12th International Symposium on Microbial Ecology. Cairns, Australia

10   Thrash, J.C., Achenbach, L.A., Coates, J.D. (2008). "A novel perchlorate-reducing alpha proteobacteria capable of mesophilic anaerobic iron oxidation." American Society for Microbiology 108th General Meeting.

11   Heinnickel, M., Smith S., Madan, S., Coates, J.D (2008). "Using perchlorate reductase from *Dechloromonas agitata* str CKB to detect perchlorate in the ppb range". 108th General Meeting American Society of Microbiology June 1-5, Boston, MA

12   Heinnickel, M.L., Smith, S., Coates, J.D. (2007) A colorimetric bioassay for perchlorate. In abstracts of the Annual Meeting of the American Geophysical Union, San Francisco, CA Dec 10th – 14th

13   Coates, J.D. (2007) The microbiology of perchlorate in the environment. In abstracts of the Annual Meeting of the American Geophysical Union, San Francisco, CA Dec 10th – 14th

14   Thrash, J.C., Achenbach, L.A., Coates, J.D. (2007). Bioelectrical perchlorate treatment. In abstracts of the Annual Meeting of the American Geophysical Union, San Francisco, CA Dec 10th – 14th

15   Sun, Y. and Coates, J.D. (2007) Effects of nitrate on microbial perchlorate reduction. In abstracts of the Annual Meeting of the American Geophysical Union, San Francisco, CA Dec 10th – 14th

16   Sun, Y. Smith, S., Chan, E., and Coates, J.D. (2007) "Regulation of Microbial Perchlorate Reduction by Nitrate". 107th American Society for Microbiology General Meeting. May 21 – 25. Toronto, Canada

17   Smith, S., Heinnickel, M. and Coates, J.D. (2007) A colorimetric bioassay for perchlorate In: Abstracts of the Partners in Environmental Technology Symposium and Workshop, Washington DC

18   Thrash, J.C., Achenbach, L.A., Coates, J.D. (2007). Continuous bioelectrical perchlorate treatment. In: Abstracts of the Partners in Environmental Technology Symposium and Workshop, Washington DC