FINAL REPORT-PHASE II

Prokaryotic cDNA Subtraction: A Method to Rapidly Identify Functional Gene Biomarkers

SERDP Project ER-1563

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14. ABSTRACT

Perchlorate is a soluble contaminant can be biologically reduced to innocuous chloride. As with any treatment process, a biological process needs multiple indicators that it is functioning appropriately. Biomarker concentration is one such indicator of process function. In this project, the utility of quantitative biomarker assays for monitoring the health of perchlorate-reducing bioreactors was demonstrated at the bench- and pilot-scale. The quantitative assays refined in this project can now be directly applied to monitor the potential for biological perchlorate reduction at a contaminated site or the transcriptional activity of perchlorate-reducing bacteria in a treatment system. Additionally, Prokaryotic cDNA Subtraction, a focused gene discovery technique, was modified for successful deployment on a perchlorate-reducing isolate; this exciting development resulted in the identification of a perchlorate-related gene sequence that has substantial differences from related, known sequences. The improved Prokaryotic cDNA Subtraction protocol can now be applied to other perchlorate-reducing bacteria and other prokaryotes that transform contaminants of interest to the Department of Defense, thereby expanding the functional gene sequence database and facilitating improved biomarker design.

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Perchlorate, molecular biological tool, perchlorate reductase, chlorite dismutase, Prokaryotic cDNA Subtraction

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List of Acronyms

ANOVA	analysis of variance
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
C_t	cycle threshold
DNA	deoxyribonucleic acid
DNT	2,4-dinitrotoluene
DoD	Department of Defense
EBCT	empty bed contact time
MBT	molecular biological tool
MG-RAST	Metagenomics Rapid Annotation using Subsystem Technology
mRNA	messenger RNA
PCR	polymerase chain reaction
PRB	perchlorate-reducing bacteria
QC	quality control
qPCR	quantitative real-time PCR
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT-qPCR	reverse-transcription, quantitative real-time PCR
SERDP	Strategic Environmental Research and Development Program
SEED	SERDP Exploratory Development
SON	Statement of Need
SSH	suppression subtractive hybridization
TNT	2,4,6-trinitrotoluene
USEPA	United States Environmental Protection Agency

Keywords

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Abstract

Perchlorate is a stable, soluble contaminant that occurs in the environment due to anthropogenic and natural activities. Some microorganisms can reduce perchlorate to innocuous chloride, and that potential can be harnessed for the biological treatment of perchlorate-contaminated waters. The discovery and application of biomarkers has the potential to improve the design, performance, and monitoring of such biological treatment processes for the removal of perchlorate and other compounds relevant to the Department of Defense.

Objectives. Building on our previous Strategic Environmental Research and Development Program Exploratory Development project (ER-1563, "Prokaryotic cDNA Subtraction: A Method to Rapidly Identify Functional Gene Biomarkers"), the purpose of the current project was to further develop molecular biological tools that are useful in assessing biodegradation potential at perchlorate-contaminated field sites and for monitoring the transcriptional activity of perchlorate-reducing bacteria in biological treatment systems. Specific objectives for the project were (1) to refine quantitative assays for genes associated with microbial perchlorate reduction and to apply quantitative assays on bench-scale and pilot-scale perchlorate-reducing bioreactors; and (2) to improve the protocol for Prokaryotic Suppression Subtractive Hybridization Polymerase Chain Reaction Complementary Deoxyribonucleic Acid Subtraction (hereafter termed Prokaryotic cDNA Subtraction), a focused gene discovery technique that was used to expand the database of perchlorate-related gene sequences for biomarker development.

Technical Approach. Building on the results of ER-1563 (Tasks 1-5), the current project was divided into seven additional tasks (Task 6-12). Tasks 6-9 focused on quantifying *cld* and *pcrA* genes or transcripts in pure cultures and in bench- and full-scale perchlorate-reducing bioreactors. Two key enzymes in the perchlorate reduction pathway are chlorite dismutase, which is encoded by the *cld* gene, and perchlorate reductase, which is encoded by the *pcrABCD* genes. Tasks 10-12 focused on improving the Prokaryotic cDNA Subtraction protocol and deploying it on a perchlorate-reducing isolate to identify perchlorate-related gene sequences.

Results. In Task 6, we re-designed the existing *cld* quantitative real-time polymerase chain reaction (qPCR) assay to produce a substantial increase in PCR amplification efficiency and to increase the minimum detectable *cld* concentration. In Task 7, the transcription of *cld* and *pcrA* was examined in a bench-scale perchlorate-reducing bioreactor. The concentration of *cld* transcripts was 2- to 8-fold lower under near-failure conditions (<25% perchlorate removal) than during normal operation (>50% perchlorate removal) in the bioreactor, which suggests that the near-failure in the bioreactor might have been due in part to a loss of perchlorate-reducing bacteria from the reactor. In Task 8, the presence of *cld* and *pcrA* was examined in two pilot-scale perchlorate-reducing bioreactors. The concentrations of *cld* were 3- to 4-fold higher in the bioreactor that removed 90% of the influent perchlorate as compared to the bioreactor that removed 63% of the influent perchlorate, which suggests that a larger perchlorate-reducing bacterial community was present in the reactor that demonstrated superior perchlorate removal. Thus, based on Tasks 7-8, it was concluded that the concentrations of *cld* genes and transcripts are likely to be a good indicator of bioreactor health. Since the data from Tasks 7 and 8 demonstrated that the concentration of *pcrA* genes and transcripts were at least one order of magnitude less than the concentration of *cld* genes and transcripts in each bioreactor, Task 9

investigated the potential for bias in the existing *pcrA* qPCR assay. The results showed that mismatches between the template and primer near the 5' end of the primer caused *pcrA* quantities to be substantially underestimated, often by one or more orders of magnitude. Thus, we designed new *pcrA* primer sets targeting several perchlorate-reducing strains: *Dechloromonas* sp. PC1, *Dechloromonas aromatica* RBC, *Dechloromonas agitata* CKB, and *Azospira* sp. KJ. Each primer set amplified *pcrA* in its target strain well but did not amplify *pcrA* from the other tested strains. Therefore, these primer sets might be deployed in parallel to more accurately quantify *pcrA* in mixed microbial communities.

Since relatively few perchlorate-related gene sequences are known, primers and probes for interrogating the potential for perchlorate reduction at a contaminated site or the transcriptional activity of perchlorate-reducing bacteria in a treatment system have been narrowly designed. Prokaryotic cDNA Subtraction is a focused gene discovery technique that can be used to identify genes that are up-regulated under a condition of interest (e.g., perchlorate-reducing conditions). Thus, this technique could be used to expand the database of gene sequences related to perchlorate reduction to facilitate improved biomarker design. Key to the successful application of the technique is avoiding the identification of ribosomal ribonucleic acid (rRNA) sequences rather than the transcripts of interest in the messenger RNA (mRNA). In Tasks 10 and 11, we demonstrated two modifications in the Prokaryotic cDNA Subtraction protocol to decrease the identification of rRNA sequences in our test organism (perchlorate-reducing strain JDS4 in the *Rhodocyclaceae* family). The first modification (sequential mRNA purification with two kits using different capture oligonucleotides for rRNA) was deployed in a Prokaryotic cDNA Subtraction on JDS4. The modified protocol was successful, and three perchlorate-related genes were identified: perchlorate reductase alpha subunit precursor, perchlorate reductase A, and perchlorate reductase B. In particular, the identified *pcrA* sequence had seven mismatches with the existing *pcrA* qPCR primer set, which means that we have taken an important first step in developing an expanded functional gene sequence database related to perchlorate reduction.

<u>Benefits</u>. The quantitative assays for *cld* and *pcrA* that were refined in this project can now be directly applied to more accurately and sensitively monitor the potential for biological perchlorate reduction at a contaminated site or the transcriptional activity of perchlorate-reducing bacteria in a treatment system; thus, these assays will be beneficial at Department of Defense sites and drinking-water treatment plants practicing biological perchlorate reduction. The successful application of Prokaryotic cDNA Subtraction to identify perchlorate-related genes means that this protocol can now be applied to other prokaryotes that transform contaminants of interest to the Department of Defense, including 1,4-dioxane, 2,4,6-trinitrotoluene (TNT), 2,4-dinitrotoluene (DNT), and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), thereby leading to new or improved biomarkers for biodegradation of these contaminants.

Our transition plan is to build upon the results of this successful project in several ways: (1) to collapse the *pcrA* primers developed in this project to a single, multiplex Taqman® assay; (2) to interrogate additional perchlorate-reducing bacteria via Prokaryotic cDNA Subtraction to continue to expand the functional gene sequence database for perchlorate reduction; (3) to quantitatively monitor *cld* and *pcrA* in the full-scale biological drinking water treatment facility in Rialto, California; and (4) to quantitatively monitor *cld* and *pcrA* expression under various applied perturbations to determine the resiliency of perchlorate-reducing bacteria.

1.0 Objectives

Statement of Need (SON) ERSON-07-03 affirmed the need for methods to identify biomarkers associated with the biodegradation of compounds relevant to the Department of Defense (DoD), such as perchlorate. Building on our previous Strategic Environmental Research and Development Program (SERDP) Exploratory Development (SEED) project (ER-1563, "Prokaryotic cDNA Subtraction: A Method to Rapidly Identify Functional Gene Biomarkers", (Kirisits *et al.* 2008), the purpose of the current project was to further develop molecular biological tools (MBTs) that are useful in assessing biodegradation potential and activity at perchlorate-contaminated field sites. Thus, the current project responds directly to SERDP's desire to improve the arsenal of MBTs for DoD-relevant pollutants.

The project had two main objectives. The first objective was to refine and apply quantitative assays for genes related to microbial perchlorate reduction. The focus was on two key genes involved in perchlorate reduction: *cld* (gene encoding chlorite dismutase) and *pcrA* (gene encoding the α-subunit of perchlorate reductase). qPCR assays were refined using pure cultures of perchlorate-reducing bacteria (PRB) and then extended to reverse transcription qPCR (RT-qPCR) assays. qPCR or RT-qPCR assays for *cld* and *pcrA* were deployed against bench-scale and pilot-scale perchlorate-reducing bioreactors. The second objective was to improve the protocol for Prokaryotic cDNA Subtraction, a focused gene discovery technique. The improvements were targeted to increase the likelihood of identifying biodegradation genes in environmental bacteria, and the improved protocol was tested on strain JDS4, a perchlorate-reducing bacterium in the *Rhodocyclaceae* family.

2.0 Background

The background section discusses 1) the microbiology of perchlorate reduction and the MBTs that are suitable for interrogating microbial perchlorate reduction, 2) available biological perchlorate treatment technologies, and 3) Prokaryotic cDNA Subtraction for biodegradation gene discovery.

2.1 Microbial perchlorate reduction

Perchlorate was selected as the model pollutant for this project because of its persistence in the environment, the paucity of functional gene sequences associated with its reduction, and the phylogenetic diversity of PRB. Perchlorate is stable and highly soluble; it contaminates groundwater easily and has even been detected in dairy milk and breast milk (Gullick *et al.* 2001, Kirk *et al.* 2005, Renner 1998, Urbansky and Brown 2003). To date, the United States Environmental Protection Agency (USEPA) has documented perchlorate releases in at least 36 states (USEPA 2005). Perchlorate is known to cause adverse health effects including disruption of thyroid function (Kirk 2006).

Due to the prevalence of perchlorate contamination, reliable and cost effective *ex situ* and *in situ* treatment methods are needed. Biological treatment is a promising approach, and many PRB have been isolated from activated sludge and environmental samples (Bruce *et al.* 1999, Coates *et al.* 1999a, Rikken *et al.* 1996, Shrout *et al.* 2005a, Waller *et al.* 2004). PRB are capable of using a wide variety of electron donors including many simple organics (e.g., acetate), hydrogen, and Fe (II) (Bruce *et al.* 1999, Coates *et al.* 1999b, Lack *et al.* 2002, Shrout *et al.* 2005b). In addition, PRB can use a number of different electron acceptors in addition to perchlorate including chlorate, oxygen, nitrate, and Mn (IV) (Bruce *et al.* 1999, Coates *et al.* 1999a, Kengen *et al.* 1999). Some strains preferentially use one electron acceptor, such as nitrate, while others appear to simultaneously reduce multiple electron acceptors (Chaudhuri *et al.* 2002, Nozawa-Inoue *et al.* 2005, Zhang *et al.* 2005).

PRB have been identified among a broad range of phylogenetic groups including the alpha, beta, gamma, and epsilon subclasses of the *Proteobacteria* (Coates *et al.* 1999b). While many applications of MBTs to biological processes have sought to determine the phylogenetic identity of the microorganisms present, this has limited utility because phylogeny is not necessarily predictive of metabolic capability (Jaspers and Overmann 2004); this is certainly the case for PRB because of their aforementioned phylogenetic diversity. Targeting functional genes (defined as genes that encode biodegradation enzymes) offers more insight into fundamental biological processes, and several functional gene targets exist for PRB. In the perchlorate-reducing microorganisms investigated thus far at the molecular level, perchlorate is reduced to chlorate and then to chlorite by perchlorate reductase (encoded by *pcrABCD*). Chlorite, a toxic by-product, is converted by chlorite dismutase (encoded by *cld*) to oxygen and chloride.

MBTs can be used to interrogate these functional genes (*pcrABCD* and *cld*) at contaminated sites and in engineered treatment processes. qPCR is an MBT that can be used to determine the number of DNA copies of a target gene in a sample. This assay is useful to assess perchlorate-reduction potential in the microorganisms present at a site; if the assay shows insufficient

numbers of perchlorate-related genes (e.g., *cld* and *pcrA*) in the native microbial community, supplementation with known PRB (i.e., bioaugmentation) might be suggested. While DNA-based MBTs are a useful first step in assessing *the potential* of a biodegradation process, only mRNA-based MBTs provide information about the genes that a microorganism is *actively transcribing*. Under a particular set of environmental conditions, microorganisms will transcribe a portion of their DNA to mRNA transcripts, and these transcripts can then be translated into functional enzymes. RT-qPCR is an MBT that can be used to determine the number of mRNA copies of a target gene transcript in a sample. This assay is useful to assess perchlorate-reduction activity at a site; if the assay shows ample perchlorate-reduction transcripts, this would suggest the success of the biodegradation process. If the assay shows insufficient perchlorate-reduction transcripts, this would suggest that changes are needed to stimulate PRB at the site (e.g., supplementation of electron donor or trace metals).

Nozawa-Inoue *et al.* (2008) developed a set of primers targeting *pcrA*, which encodes the catalytic subunit of perchlorate reductase, and detected this gene in pure cultures and soil samples. These primers were developed from only two gene sequences, so it was not known how well this primer set covers the diversity of *pcrA* sequences that might exist in other PRB. Nozawa-Inoue *et al.* (2008) also developed *Escherichia coli* plasmids containing the cloned *pcrA* gene from *Dechloromonas agitata* strain CKB to be used as a standard for quantifying *pcrA* via qPCR. Some bacteria possess *pcr* but not *cld*. These bacteria cannot survive in the presence of high perchlorate because chlorite will build up to toxic levels (Bender *et al.* 2004). Therefore, monitoring *pcr* genes alone is not a clear predictor of perchlorate-reducing capability.

Two sets of primers for detection of *cld* exist (Bender *et al.* 2004), but they are based on limited sequence information (i.e., nine sequences for one primer set and four sequences for the other primer set). Thus, it was not known how well these primer sets cover the diversity of chlorite dismutase sequences that might exist in other PRB. It is possible for a strain to contain *cld* but be unable to reduce perchlorate (Bender *et al.* 2004). Therefore, monitoring *cld* alone also is not a clear predictor of perchlorate-reducing capability.

2.2 Biological perchlorate treatment processes

PRB use perchlorate as an electron acceptor, reducing perchlorate under anaerobic conditions. An energy source (e.g., acetate) must be provided. A number of different bioreactor configurations have been evaluated and some have been demonstrated at the pilot- and full-scale (Sutton 2006). In laboratory- and pilot-scale reactors, perchlorate has been treated *ex situ* using fluidized bed reactors, fixed-bed reactors, and moving-bed biofilm reactors (Brown *et al.* 2005, De Long *et al.* 2012, Logan *et al.* 2001).

Effective reactor design must consider the source and ultimate use of the perchlorate-contaminated water, the influent perchlorate concentration, and the concentration of competing electron acceptors (i.e., oxygen and nitrate). A suitable electron donor must be provided to reduce all or nearly all competing electron acceptors and perchlorate. For drinking water treatment, oxygen and nitrate concentrations typically drive the required concentration of electron donor because they are present in much higher concentrations (mg/L), and perchlorate is typically present at low levels (μg/L). A number of different organic electron donors have been

used successfully including acetate, ethanol, lactate, and pyruvate (Brown *et al.* 2002, Brown *et al.* 2003, Choi *et al.* 2007, Logan 2001, Min *et al.* 2004), and the inorganic electron donor hydrogen also has been shown to support biological perchlorate reduction (Logan 2001, Logan and LaPoint 2002).

Although the feasibility of biological perchlorate treatment has been demonstrated, barriers still exist (e.g., the risk of process upsets and failures) that prevent its widespread application. Fixed-bed reactors require periodic backwashing to remove biomass and prevent filter clogging. Upsets can be caused by over-backwashing (Choi *et al.* 2007), changes in influent water characteristics, or disruptions to the primary substrate feed. Research is needed to understand how the presence and expression of functional genes related to perchlorate reduction respond to varying influent water characteristics, operational changes, and process upsets. Deployment of MBTs for these purposes would serve to reduce the risks associated with the application of biological perchlorate treatment.

To develop reliable biomarkers that can be used in the field to assess natural attenuation potential or to monitor engineered biological perchlorate treatment systems, the database of functional genes involved in perchlorate reduction must be expanded. Prokaryotic cDNA Subtraction is an MBT for functional gene discovery. By applying this tool to PRB from various taxonomic groups with different kinetic attributes, identification of gene sequences with significant divergence from known perchlorate-reduction genes can be explored.

2.3 Prokaryotic cDNA Subtraction

As noted above, target gene sequences are required for primer design for qPCR and RT-qPCR assays. The complete gene sequences encoding perchlorate reductase (*pcrABCD*) and chlorite dismutase (*cld*) are known for four strains of PRB (Table 1). For a number of other strains, only partial gene sequences or no gene sequences related to perchlorate reduction are available (Bender *et al.* 2002, Bender *et al.* 2005, Coates *et al.* 1999b, Nozawa-Inoue *et al.* 2008).

Table 1. Gene sequences related to perchlorate reduction

Selected strains	***cld* gene sequence***	***pcrA* gene sequence****
Dechloromonas aromatica RCB	complete genome sequence	
Dechlorosoma suillum PS	complete genome sequence	
Dechloromonas agitata CKB	complete	complete
Dechlorosoma sp. KJ (now called *Azospira* sp. KJ (Steinberg *et al.* 2005))	complete	complete
Dechloromarinus chlorophilus NSS	partial	none
Dechlorospirillum sp. WD	partial	partial
Pseudomonas sp. PK	partial	none
Azospirillum sp. TTI	none	partial
Rhodocyclaceae strain JDS4	none	None

The *Dechloromonas aromatica* and *Dechlorosoma suillum* genomes are available online (http://www.ncbi.nlm.nih.gov/).
* Bender *et al.* (2004, 2002)
** Nozawa-Inoue *et al.* (2008); Bender *et al.* (2005)

cDNA Subtraction is an attractive method for the identification of environmentally relevant genes because it amplifies only those genes that are specifically expressed under one chosen set of conditions (e.g., perchlorate-reducing conditions). cDNA Subtraction was previously developed for application to eukaryotes (Diatchenko *et al.* 1996, Plum and Clarkcurtiss 1994, Sugimoto *et al.* 2004) due to the ease of isolating eukaryotic mRNA through its polyA tail. We successfully extended the technique for application to prokaryotes (De Long *et al.* 2008), whose mRNA lacks this polyA tail.

The Prokaryotic cDNA Subtraction method is summarized in Figure 1. Bacteria are grown with and without a particular pollutant, perchlorate in the current project; these cultures are called the tester and driver, respectively. (In our previous development of this technique (De Long *et al.* 2008), the pollutant was toluene (as shown in Figure 1)). From these cultures, mRNA is isolated and double-stranded cDNA is synthesized. The cDNA is digested; one portion of tester cDNA is ligated to adaptor 1, and another portion is ligated to adaptor 2 (Figure 1, step I). The tester cDNA pools are heat-denatured and hybridized with an excess of heat-denatured driver cDNA (Figure 1, step II). These pools are mixed, more heat-denatured driver cDNA is added in a subsequent hybridization, and the overhang ends are filled in by DNA polymerase (Figure 1, step III). Tester 1 cDNA fragments that were single-stranded after the first hybridization can now hybridize to their complements in the tester 2 pool. This yields double-stranded cDNA fragments with adaptor 1 and 2 on opposite ends (type a in Figure 1, step III); the fragments represent genes that are differentially expressed in the absence and presence of the pollutant of interest, and they are amplified in the suppression PCR step using primers that are complementary to adaptors 1 and 2 (Figure 1, step IV). Other types of fragments are not amplified. For instance, fragments that have adaptors only on one end or no adaptors at all (e.g., types b, e, f, and g in Figure 1, step III) are not amplified. Additionally, fragments with the same adaptor on both ends (types c and d in Figure 1, step III) cannot be amplified because the adaptor on the 5’ end hybridizes to its complement on the 3’ end. After suppression PCR, the amplicon is sequenced.

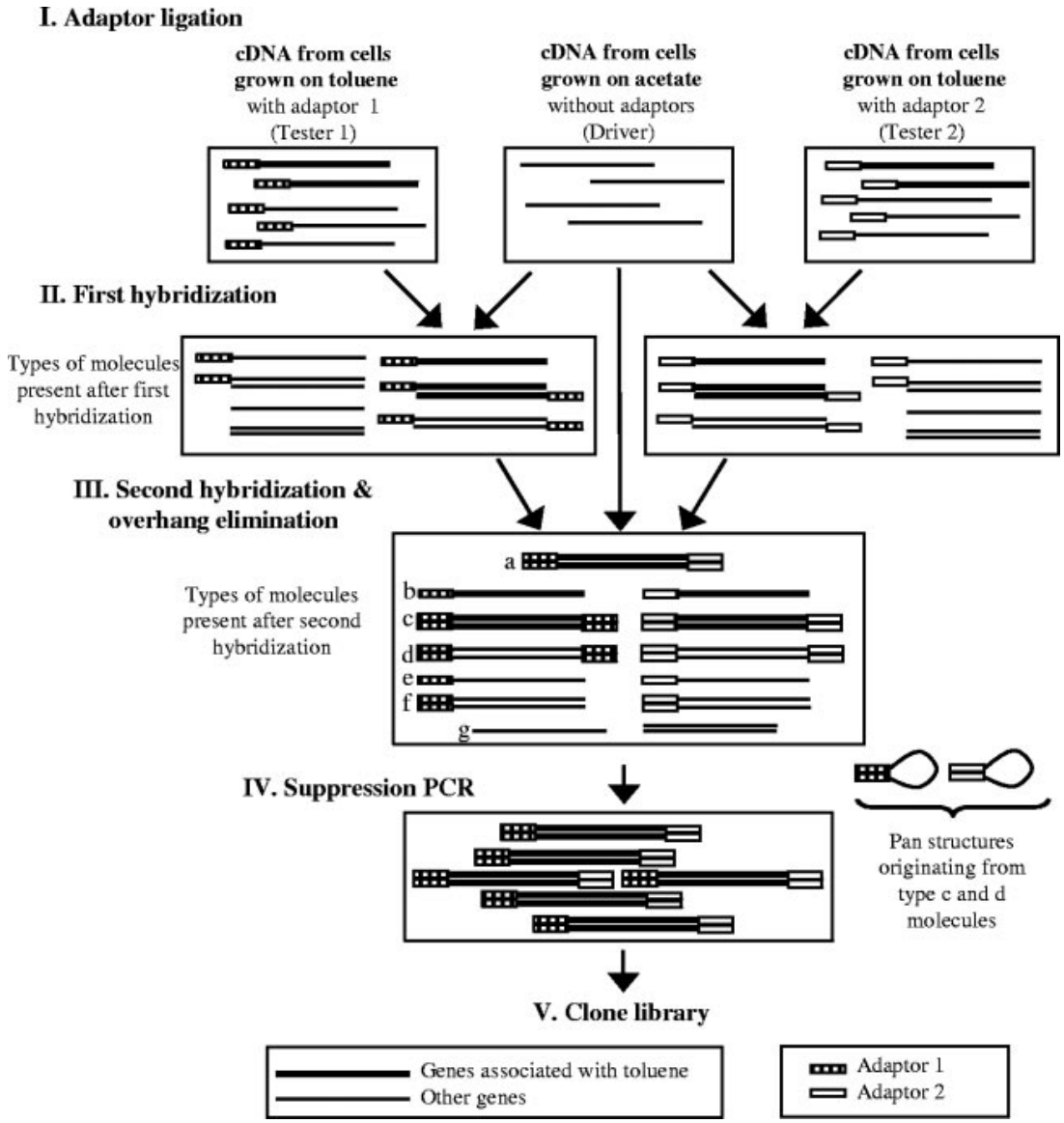

Figure 1. Schematic of Prokaryotic cDNA Subtraction
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3.0 Materials and Methods

To meet the objectives of the study, the project was divided into seven tasks. Since this work is a continuation of a previous SEED project from SERDP ("Prokaryotic cDNA Subtraction: A Method to Rapidly Identify Functional Gene Biomarkers", Kirisits *et al.* 2008), the numbering of tasks begins with Task 6. The Materials and Methods are discussed for each objective and task.

3.1 Objective 1: Refine and apply quantitative assays targeting genes related to microbial perchlorate reduction

To meet this objective, four tasks were completed. In Task 6, we refined a quantitative assay for *cld*. The qPCR assay refined in this task was used to quantify the number of *cld* copies in DNA from PRB. In Task 7, we deployed quantitative assays on a bench-scale perchlorate-reducing bioreactor. The existing RT-qPCR assay for *cld* (De Long *et al.* 2010) and the existing *pcrA* qPCR assay (Nozawa-Inoue *et al.* 2008) was extended to RT-qPCR, and these assays were used to interrogate a bench-scale perchlorate-reducing bioreactor to see how the *cld* and *pcrA* concentrations in the mRNA changed as a function of water quality conditions. In Task 8, we deployed these same qPCR assays on DNA from a field perchlorate-reducing bioreactor. In Task 9, we addressed the potential bias of the existing *pcrA* qPCR assay (Nozawa-Inoue *et al.* 2008). The methods associated with each of these tasks are outlined as follows.

3.1.1 Materials and methods related to objective 1

<u>Task 6. Refine quantitative assay for *cld*</u>

In our previous SEED project, we developed a *cld* qPCR assay using existing endpoint *cld* primers (UCD-238F and UCD-646R) developed by Bender *et al.* (2004). As described in De Long *et al.* (2010), the qPCR reactions contained 1X Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 0.4 µM of each primer, and 5 ng DNA. qPCR reactions were run on a ViiA™ 7 Real-Time PCR system (Applied Biosystems, Foster City, CA). The thermocycling program was as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles: 95°C for 15 s, 50°C for 45 s, and 60°C for 60 s. A final melting (dissociation) curve analysis was performed to verify the specificity of the amplified products. A standard curve was generated using *Dechloromonas aromatica* RCB genomic DNA. The PCR amplification efficiency was calculated from the slope of the standard curve.

To refine the *cld* qPCR assay of De Long *et al.* (2010), *cld* sequences from seven PRB and four chlorate-reducing bacteria were obtained from Genbank (Table 2) to design new primers. The nucleotide sequences between the UCD-238F and UCD-646R target regions were visualized by manually trimming and aligning the retrieved sequences using Geneious Pro 5.1.4 (Biomatters Ltd). Primer sequences were selected by the Primer 3 program (Rozen and Skaletsky 2000). The sequences were manually compared, and a degenerate base was added to a candidate primer position as appropriate. From these, we chose six candidate primers to use in conjunction with the existing forward (UCD-238F) or reverse (UCD-646R) primer to produce an amplicon in the optimal size range for qPCR (Table 3).

Table 2. Bacterial strains used for re-designing *cld* primers

Strain	GenBank Accession No.
Pseudomonas chloritidismutans	EU436747
Dechloromonas hortensis	EU436749
Pseudomonas sp. PK	AY540958
Dechloromarinus chlorophilus	AY540959
Azospira oryzae	AY540960
Azospira sp. KJ	EU571095
Dechloromonas aromatica RCB	AAZ47311
Ideonella dechloratans	AJ296077
Dechloromonas sp. LT-1	AY540957
Dechloromonas agitata CKB	AY124796
Dechlorospirillum sp. WD	AY540961

Table 3. qPCR primers designed for *cld*

Candidate Primers	Sequence (5' to 3')	Nucleotide Positions of *D. agitata*	Forward or Reverse	Theoretical Amplicon Size (bp) [a]
UTX-340R	TCV MBC ADR AAN KBC TGB GH	340 to 360	Reverse	120
UTX-353R	HGT SGM VCG VMA DTC VMB CA	353 to 373	Reverse	140
UTX-544F	TGG TGG AAY MTK WCS SMB GMR SA	544 to 567	Forward	100
UTX-454F	CCN RRB CTG AAY RRB GGH CT	454 to 474	Forward	194
UTX-451R	AGD CCV YYR TTC AGV YYN GG	451 to 471	Reverse	210
UTX-518F	YSW TYR TVA THC CVG TSA ARA	518 to 538	Forward	150

[a] Amplicon sizes in base pairs (bps) were calculated based on combining each candidate primer with the appropriate existing forward or reverse primer (i.e., UCD-238F or UCD-646R).

Variations in the primer concentrations and thermal cycler profiles were tested with the candidate primer sets. Primer concentrations of 0.4, 0.6 and 1 µM were used. Two sets of thermal profiles were used; thermal cycler profile A: 50°C for 2 min, 95°C for 5 min, followed by 95°C for 15 s and 60°C for 1 min (40 cycles) and thermal cycler profile B: 50°C for 2 min, 95 °C for 5 min, followed by 95°C for 15 s, 50°C for 20 s, and 60°C for 40 s (40 cycles). The primer sets were tested on DNA from several isolates: *Dechloromonas aromatica* RCB, *Dechloromonas agitata* CKB, *Azospira* sp. KJ, *Dechloromonas* sp. PCC, *Dechloromonas* sp. PC1, *Dechlorospirillum* sp. AEO1, *Dechlorospirillum* sp. AEO2, *Dechlorospirillum* sp. AEO3, *Dechlorospirillum* sp. AEO4, *Acinetobacter* sp. BD413, and *Pseudomonas aeruginosa* PAO1. A standard curve was generated with 10-fold serial dilutions of known amounts of genomic DNA from *Dechloromonas aromatica* RCB.

Task 7. Deploy quantitative assays on a bench-scale perchlorate-reducing bioreactor

As described in De Long *et al.* (2012), a one-inch inner-diameter bench-scale bioreactor was constructed, using 2-mm etched glass beads as packing material. The total bed depth was 4 inches, with a liquid-phase sampling port for each 1-inch section of the bed. The bioreactor was

operated in up-flow mode with a 5-min empty bed contact time (EBCT). The bioreactor was fed synthetic groundwater containing 100 μg/L perchlorate and sufficient acetate to remove the dissolved oxygen, nitrate, and perchlorate from the water. The bioreactor was inoculated with a perchlorate-reducing enrichment culture developed in the previous SEED project (Kirisits *et al.* 2008). To monitor bioreactor performance for perchlorate reduction, liquid samples were collected from the influent, effluent, and intermediate sampling ports. Biomass samples (consisting of approximately 150 glass beads) were collected from each 1-inch section of the bioreactor on one day during normal operation (defined as >50% perchlorate removal) and on one day during near-failure (defined as <25% perchlorate removal) conditions. Cells were lysed in Lysing Matrix E tubes with the Fast Prep 24 system (MP Biomedicals, Solon, OH), and RNA was isolated with the RiboPure™-Bacteria Kit (Ambion, Austin, TX). cDNA synthesis was conducted as described in De Long *et al.* (2010). The cDNA was used to run RT-qPCR assays for *cld* and *pcrA* according to the methods specified in De Long *et al.* (2010) and Nozawa-Inoue *et al.* (2008).

Task 8. Deploy quantitative assays on a field perchlorate-reducing bioreactor

As described in De Long *et al.* (2012), fixed-bed perchlorate-reducing pilot-scale bioreactors previously operated in Rialto, California by Carollo Engineers (ER-0544) were interrogated for the presence of *cld* and *pcrA.* To monitor bioreactor performance for perchlorate reduction, liquid samples were collected from the influent, effluent, and intermediate sampling ports of bioreactors F120 and F130. The bioreactors were cored to obtain biomass samples with depth. Lysing Matrix E tubes were used for cell lysis with the Fast Prep 24 system (MP Biomedicals, Solon, OH), and DNA was extracted with the FastDNA Spin Kit (MP Biomedicals, Solon, OH). The existing *cld* qPCR assay (De Long *et al.* 2010) and the existing *pcrA* qPCR assay (Nozawa-Inoue *et al.* 2008) were used to quantify gene concentrations.

Task 9. Address potential bias in the quantitative assay for *pcrA*

The perchlorate-reducing strains shown in Table 4 were anaerobically grown in serum bottles with 100 mM perchlorate at 35°C. The cells were harvested with a 0.22-μm filter, and DNA was extracted using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH). DNA concentrations were measured with the Quant-iT™ double-stranded DNA Assay kit (Invitrogen, Carlsbad, CA) for accurate template quantification. The existing *pcrA* qPCR assay with primers pcrA320F and pcrA598R (Nozawa-Inoue *et al.* 2008) was performed on this DNA using a ViiA™ 7 Real-Time PCR system (Applied Biosystems, Foster City, CA) with the following thermal cycler profile: 50°C for 2 min; 95°C for 10 min; 40 cycles: 95°C for 15 s and 60°C for 60 s. Each reaction contained 2 μL of template DNA, 5 μL of SYBR Green I PCR Master Mix (Applied Biosystems, Foster City, CA), 0.2 μM of each primer, and 1 μL 10×BSA (bovine serum albumin).

Table 4. Mismatches between *pcrA* primers and *pcrA* genes of selected PRB (Table modified from De Long *et al.* 2010)

Isolate	Number of mismatches between the strain's *pcrA* sequence and the stated *pcrA* primers		Genbank accession for *pcrA* sequence
	pcrA320F	pcrA598R	
Dechloromonas aromatica RCB	2	2	CP000089
Dechloromonas agitata CKB	1	3	AY180108
Azospira sp. KJ	5	3	EU571095
Dechloromonas sp. PC1	4	3	EU022026
Rhodocyclaceae strain JDS4	unknown	unknown	

Given the mismatches between the existing *pcrA* primers (Nozawa-Inoue *et al.* 2008) and these PRB (Table 4), we designed additional *pcrA* primer sets to be perfect matches for each PRB (Table 5). The red letters indicate the presence of a nucleotide different from that in primer pcrA320F or pcrA598R (Nozawa-Inoue *et al.* 2008). The qPCR reaction and thermal cycler conditions were the same as described previously in this section. Standard curves were generated with *D. aromatica* genomic DNA and the previously amplified *pcrA* gene of *D. agitata* CKB on a plasmid (Nozawa-Inoue *et al.* 2008) with the primer sets shown in Table 5. DNA was added to qPCR reactions to produce *pcrA* concentrations of $6x10^6$ and 6.5×10^9 per reaction for *D. aromatica* genomic DNA and the *pcrA* plasmid, respectively. The bacteria shown in Table 5 were interrogated with these primer sets.

Table 5. qPCR primer sets designed for *pcrA**

Seq Name	Label	Sequence (5' – 3')	Strain perfectly matched to stated primer
pcrA320F	pcrA0	GCG CCC ACC ACT ACA TGT AYG GNC C	(Nozawa-Inoue *et al.* 2008)
182F	pcrA1	GCG CAC ACC ACT ACA TGT ATG GTC C	*Dechloromonas agitata* CKB
183F	pcrA2	GTG CCC ACG ACT ACA TGT ATG GCC C	*Dechloromonas aromatica* RCB
185F	pcrA3	GTG GTC ACG ACT ATA TGT ATG GAC C	*Azospira* sp. KJ
186F	pcrA4	GTG GTC ACT ACT ACA TGT ATG GGC C	*Dechloromonas* sp. PC1
pcrA598R	pcrA0	GGT GGT CGC CGT ACC ART CRA A	(Nozawa-Inoue *et al.* 2008)
182R	pcrA1	GAT GGT CAC TGT ACC AGT CAA A	*Dechloromonas agitata* CKB
183R	pcrA2	GGT GAT CGC CAT ACC AGT CGA A	*Dechloromonas aromatica* RCB
185R	pcrA3	GGT GAT CGC CAT ACC AAT CGC A	*Azospira* sp. KJ
186R	pcrA4	GAT GAT CAC CGT ACC AGT CGA A	*Dechloromonas* sp. PC1

*The red bases in the table indicate the presence of a nucleotide different from that in the existing *pcrA* qPCR primer set (pcrA320F and pcrA598R (Nozawa-Inoue *et al.* 2008)).

3.2 Objective 2: Improve the Prokaryotic cDNA Subtraction protocol for application to environmental bacteria

To meet this objective, three tasks were completed. In Task 10, we tested mRNA purification methods to increase rRNA removal in the Prokaryotic cDNA Subtraction protocol. In Task 11, we tested a modified hybridization step to improve the recovery of cDNA related to functional gene transcripts as opposed to rRNA. In Task 12, we deployed the improved Prokaryotic cDNA Subtraction protocol on PRB strain JDS4 to identify perchlorate-related genes.

3.2.1 Materials and methods related to objective 2

Task 10. Increase rRNA removal in the Prokaryotic cDNA Subtraction protocol

Perchlorate-reducing strain JDS4 was grown anaerobically on bicarbonate-buffered basal medium containing mineral and vitamin solutions at 30°C (Thrash *et al.* 2010). DNA was extracted using the UltraClean® Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA). The 16S rRNA and 23S rRNA genes were amplified via end-point PCR (Hunt *et al.* 2006, Liu *et al.* 1997) using touchdown PCR (Korbie and Mattick 2008) and were sequenced at the Institute for Cellular and Molecular Biology at the University of Texas at Austin. Total RNA was extracted using the Ribopure™-Bacteria Kit (Ambion, Austin, TX). The manufacturer of the MICROBexpress™ kit (Ambion, Austin, TX), which is used to purify mRNA from total RNA in the existing Prokaryotic cDNA Subtraction protocol (De Long *et al.* 2008), examined the complementarity between the capture oligonucleotides in the kit and the 16S and 23S rRNA sequences of strain JDS4; the capture oligonucleotides in the kit were perfectly complementary to the 16S rRNA sequence of JDS4 but not to its 23S rRNA sequence. Custom oligonucleotides for improved rRNA removal from JDS4 total RNA were unavailable in conjunction with the MICROBexpress™ kit. However, the capture oligonucleotides in the Ribo-Zero™ rRNA removal kit (Epicentre®, Madison, WI) were perfectly complementary to the 16S and 23S rRNA sequences of strain JDS4.

Thus, to increase rRNA removal in the Prokaryotic cDNA Subtraction protocol, two modifications were evaluated. The first modification used three rounds of mRNA purification with the MICROBexpress™ kit as compared to the two rounds of mRNA purification with the MICROBexpress™ kit in the existing Prokaryotic cDNA Subtraction protocol (De Long *et al.* 2008). The second modification used one round of mRNA purification with the MICROBexpress™ kit (Ambion, Austin, TX) followed by one round with the Ribo-Zero™ rRNA removal kit (Epicentre®, Madison, WI).

The efficacy of these modifications to the rRNA removal process was tested using an RT-qPCR assay targeting the 16S and 23S rRNA. Total RNA and mRNA were isolated from strain JDS4 that had been grown anaerobically. Triplicate qPCR reactions were run on an Applied Biosystems Viia™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA) using the One Step SYBR Ex Taq RT-PCR kit (Clontech Laboratories Inc, Mountain View, CA). Total RNA and mRNA were amplified with the qPCR primer sets for 16S (Li *et al.* 2010) and 23S rRNA genes (Hunt *et al.* 2006). The percent removal of 16S and 23S rRNA was calculated for the two modifications to the mRNA purification protocol described in the previous paragraph.

Task 11. Modify the hybridization step in the Prokaryotic cDNA Subtraction protocol

A synthetic pool of tester and driver cDNA molecules, consisting of defined mixtures of DNA amplicon produced from the 16S rRNA, 23S rRNA, *cld*, and *pcrA* genes, was used to test modifications to the hybridization step in the Prokaryotic cDNA Subtraction protocol to improve the recovery of transcripts related to functional genes (i.e., non-rRNA-related genes). The rRNA concentration in the driver was purposefully skewed to be substantially higher than that in the

tester; the impact of this on the recovery of the functional genes *cld* and *pcrA* was tested using the PCR-Select™ cDNA Subtraction Kit (Clontech Laboratories Inc, Mountain View, CA).

The synthetic tester and driver were constructed using amplicon produced with primers for the 16S rRNA (8F and 1492R), 23S rRNA (129F and 2490R), *cld* (Bender *et al.* 2004), and *pcrA* (Nozawa-Inoue *et al.* 2008) genes. The synthetic tester cDNA pool was designed to contain much lower concentrations of 16S rRNA and 23S rRNA genes as compared to *cld* and *pcrA* (Table 6). The driver pool contained concentrations of 16S rRNA and 23S rRNA genes that were substantially higher than those found in the tester pool (Table 6). In addition, the tester pool contained concentrations of *cld* and *pcrA* that were approximately 2 orders of magnitude as high as those found in the driver pool.

Table 6. Defined mixtures of 16S rRNA, 23S rRNA, *cld* and *pcrA* genes in the constructed tester and driver pools

Constructed Tester DNA (genes/μL)				Constructed Driver DNA (genes/μL)			
16S	23S	*cld*	*pcrA*	16S	23S	*cld*	*pcrA*
1.14×10^{8}	2.44×10^{7}	1.22×10^{9}	2.51×10^{9}	6.29×10^{9}	1.03×10^{9}	1.67×10^{7}	4.72×10^{7}

Task 12. Deploy modified Prokaryotic cDNA Subtraction protocol on PRB strain JDS4

Six replicate batch cultures of JDS4 were grown in serum bottles under anaerobic conditions in bicarbonate-buffered basal medium with 100 mM perchlorate 30ºC for 3 weeks. Additional perchlorate and acetate were injected to the serum bottles aseptically every 5 days. Then, half of the cultures were maintained under perchlorate-reducing conditions, and half were switched to aerobic conditions (tester and driver, respectively). mRNA was isolated from each culture according to the modified procedures described in Task 10. From here, our previously published protocol (De Long *et al.* 2008) for Prokaryotic cDNA Subtraction was employed from first-strand cDNA synthesis through suppression nested PCR to produce amplicon for sequencing (i.e., amplicon that represents genes up-regulated under perchlorate-reducing conditions). After suppression PCR, the amplicon was processed via barcoded, multiplexed pyrosequencing (Research and Testing Laboratories Inc., Lubbock TX) on a 454 FLX+ Titanium Genome Sequencer (454 Life Sciences, Branford, CT). The barcoded primers were designed based on the sequences of nested PCR 1 and 2 primers from the Eukaryotic cDNA Subtraction kit (Clontech Laboratories, Mountain View, CA). The sequences were processed through the MG-RAST (Metagenomics Rapid Annotation using Subsystem Technology, v3.1) server at Argonne National Laboratory (http://metagenomics.nmpdr.org), providing quality control (QC) measures and taxonomic and functional classification for the sequences, as well as the ability to perform phylogenetic and metabolic reconstructions with existing databases such as Greengene and Genbank (Meyer *et al.* 2008).

4.0 Results and Accomplishments

4.1 Results from Task 6 (Refine quantitative assay for *cld*)

The goal of this task was to improve the *cld* qPCR assay. The existing *cld* primers were designed for end-point PCR (Bender *et al.* 2004), and they demonstrated a low amplification efficiency in qPCR (De Long *et al.* 2010) likely due to the 400-bp size of the amplicon. Amplicon from qPCR generally should be 100-200 bp (Wong and Medrano 2005). We designed six candidate primers to use in conjunction with the existing forward or reverse *cld* primers (UCD-238F and UCD-646R from Bender *et al.* 2004) to produce an amplicon in the optimal size range for qPCR (Table 3). The number of wobbles in the existing *cld* primers were 40% and 30% for forward and reverse primers, respectively, but the degeneracy of the new primers was up to 50%. In general, primer degeneracies up to 40% are observed in the literature. Primer degeneracies up to 50% have been reported, but such primers should be used with caution (Linhart and Shamir 2005).

Every new qPCR assay must be critically evaluated before application to environmental samples. Developing a standard curve using a defined template has been suggested for analyzing a new qPCR assay (Wong and Medrano 2005) because the slope, y-intercept, and coefficient of determination (R^2 value) can be used to assess the amplification efficiency, sensitivity, and accuracy of the assay. The candidate *cld* assays using primer sets UTX-454F+UCD-646R and UTX-544F+UCD-646R were the only ones that yielded substantial amplification among the six primer sets tested. These two primer sets were used in optimization studies, with various primer concentrations (0.4-1.0 µM) and different thermal cycler profiles (A and B, see section 3.1.1), to construct standard curves. Table 7 summarizes the slope, amplification efficiency, y-intercept, and R^2 value for each condition tested. Primer set UTX-454F and UCD-646R with primer concentrations of 0.6 µM and thermal cycler profile B was chosen as the final *cld* qPCR assay. As shown in Table 7, this assay yielded a reasonable amplification efficiency (108%) and a high R^2 value (0.98). Additionally, it had a y-intercept of ~40 (Table 7), meaning that one *cld* copy could be detected in a qPCR reaction since 40 amplification cycles were run in the thermal cycler profile.

Table 7. Optimization of qPCR conditions for two of the candidate *cld* primers

qPCR assays	Primer conc.	Thermal cycler profile	Slope	Amplification efficiency (%)	y-intercept	R^2 value
UTX-544F	0.4 µM	A	-4.04	76.8	37.740	0.76
+	0.6 µM		-3.16	107.2	43.506	0.99
UCD-646R	1.0 µM		-3.52	92.4	41.391	0.99
UTX-454F	0.4 µM		-3.98	78.3	42.620	0.81
+	0.6 µM		-3.06	112.2	39.582	0.95
UCD-646R	1.0 µM		-3.45	94.9	37.077	0.98
UTX-544F	0.4 µM	B	-4.01	77.6	45.542	0.99
+	0.6 µM		-2.91	120.6	39.286	0.98
UCD-646R	1.0 µM		-3.48	93.8	40.872	0.99
UTX-454F	0.4 µM		-3.94	79.4	47.750	0.99
+	0.6 µM		-3.14	108.2	40.229	0.98
UCD-646R	1.0 µM		-3.37	98.2	41.055	0.98

When compared to the existing *cld* qPCR assay (De Long *et al.* 2010), this refined *cld* qPCR assay had a higher PCR amplification efficiency and could quantify lower template concentrations when comparing cycle threshold (C_t) values (Figure 2).

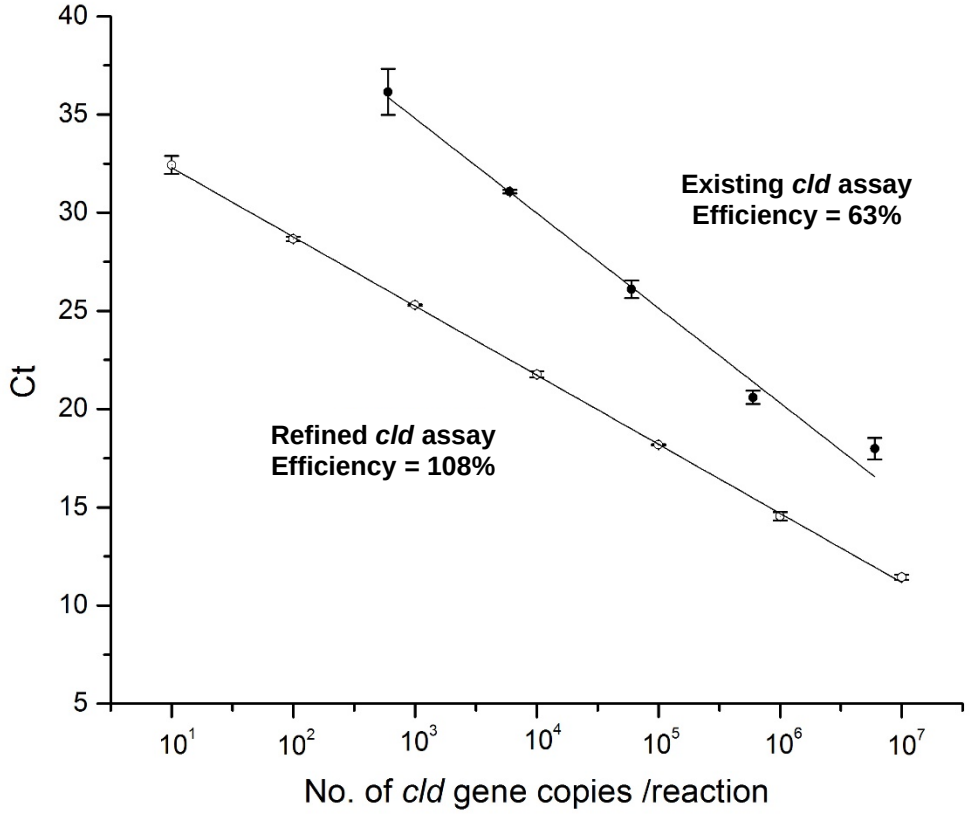

Figure 2. Comparison of the existing *cld* qPCR assay (De Long *et al.* 2010) and the refined *cld* qPCR assay using primer set UTX-454F and UCD-646R (0.6 µM primers and thermal cycler profile B)

Table 8 shows that the existing *cld* qPCR assay (De Long *et al.* 2010) and the refined *cld* qPCR assay (with primer set UTX-454F + UCD-646R) detected *cld* in known perchlorate-reducing strains but did not detect it in non-perchlorate-reducing strains. The ANOVA (analysis of variance) test showed that there was significant difference between the gene concentrations for *Dechloromonas aromatica* RCB (p-value = 0.03) in the two assays due to lower amplification efficiency in the existing *cld* qPCR assay. However, in general, the refined *cld* assay measured *cld* concentrations that were of the same order of magnitude as those measured by the existing *cld* qPCR assay for the *cld* concentration spiked in this experiment (Table 8).

Table 8. Comparison of the specificity of the existing *cld* qPCR assay (De Long *et al.* 2010) and the refined *cld* qPCR assay (UTX-454F and UCD-646R, 0.6 µM primers, and thermal cycler profile B)

Strain	qPCR assays[a]		
	Refined *cld* assay (UTX-454F + UCD-646R)	Existing *cld* assay (UCD-238F + UCD-646R)	p-value
Perchlorate-reducing bacteria			
Dechloromonas aromatica RCB	5.73 ±0.43	5.42 ±0.19	< 0.05
Dechloromonas agitata CKB	4.11 ±0.83	4.66 ±0.12	0.96
Azospira sp. KJ	6.06 ±0.34	6.76 ±0.45	< 0.05
Dechloromonas sp. PCC	5.60 ±0.25	5.44 ±0.32	< 0.05
Dechloromonas sp. PC1	4.87 ±0.18	5.01 ±0.46	< 0.05
Dechlorospirillum sp. AEO1	4.73 ±0.25	4.77 ±0.47	0.51
Dechlorospirillum sp. AEO2	3.90 ±0.87	4.76 ±0.51	< 0.05
Dechlorospirillum sp. AEO3	3.29 ±0.92	4.43 ±0.54	< 0.05
Dechlorospirillum sp. AEO4	4.80 ±0.82	4.26 ±0.21	0.26
Non-perchlorate-reducing bacteria			
Acinetobacter sp. BD413	Non-detect	Non-detect	
Pseudomonas aeruginosa PAO1	Non-detect	Non-detect	

[a] The values listed indicate the mean $\log_{10}$ copy number of *cld* per 1 ng of total DNA in the qPCR reaction ± standard deviations, calculated from serial dilutions.

In summary, the refined *cld* qPCR assay (UTX-454F + UCD-646R) produced a shorter DNA amplicon as compared to the existing *cld* qPCR assay, which substantially improved the amplification efficiency of the assay and allowed quantification of lower gene concentrations.

4.2 Results from Task 7 (Deploy quantitative assays on a bench-scale perchlorate-reducing bioreactor)

The goal of this task was to extend the use of the existing qPCR assays for *cld* (De Long *et al.* 2010) and *pcrA* (Nozawa-Inoue *et al.* 2008) to RT-qPCR assays for the interrogation of a bench-scale perchlorate-reducing bioreactor under normal and near-failure conditions.

Transcripts of *pcrA* and *cld* were detected in the bioreactor under both normal operating conditions and near-failure conditions, even in bioreactor sections where perchlorate removal was not occurring at the time of sampling (Figure 3). The quantity of *pcrA* transcripts was two to three orders of magnitude lower than the quantity of *cld* transcripts in the bioreactor; given the paucity of sequences from which the *pcrA* primers were designed, it might be that the *cld* primers were detecting a greater fraction of the PRB in this mixed microbial community. During near-failure conditions, the number of *cld* copies was two- to eight-fold lower than during

normal operation, which suggests that the near-failure in the bioreactor might have been due in part to a loss of PRB. Thus, the concentration of *cld* transcripts in a bioreactor is likely to be a good indicator of bioreactor health.

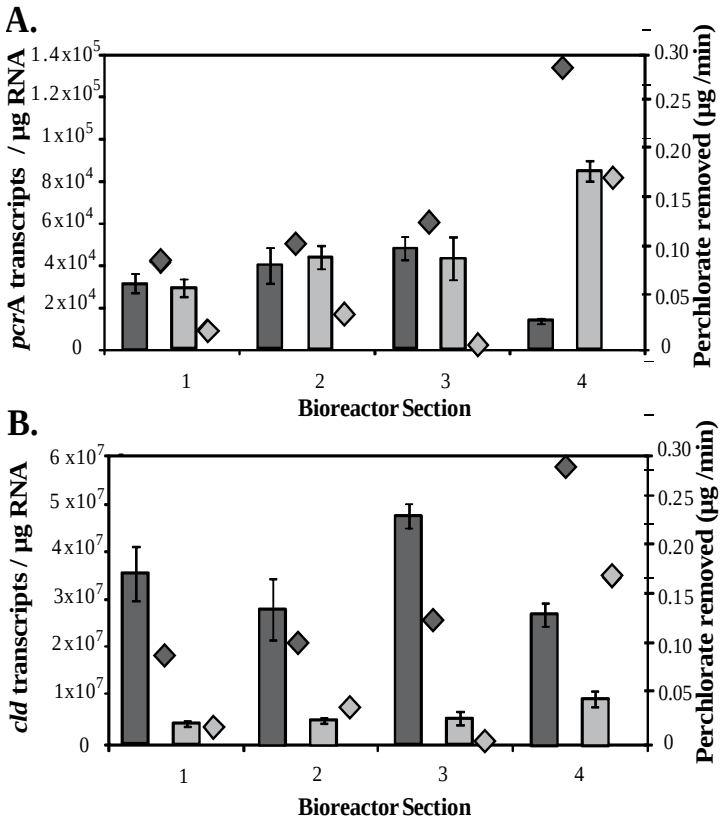

Figure 3. *pcrA* and *cld* transcript concentrations as a function of depth in the bench-scale bioreactor. A. *pcrA* transcript concentrations and perchlorate removal during normal operation (dark gray bars and diamonds, respectively) and near-failure conditions (light gray bars and diamonds, respectively). B. *cld* transcript concentrations and perchlorate removal during normal operation and near-failure conditions. [Copyright © John Wiley & Sons, Inc., De Long *et al.*, *J Appl Microbiol.* 112(3):579-592, 2012]

4.3 Results from Task 8 (Deploy quantitative assays on a field perchlorate-reducing bioreactor)

The purpose of this task was to demonstrate the existing qPCR assays for *cld* (De Long *et al.* 2010) and *pcrA* (Nozawa-Inoue *et al.* 2008) on pilot-scale fixed-bed bioreactors used for biological perchlorate reduction.

During the pilot-scale optimization period, bioreactor F120 showed superior performance to bioreactor F130 despite the fact that both bioreactors were operated at the same groundwater well with acetate as the electron donor, and F120 was operated at a shorter EBCT than was F130. At the time that the chemical profile data were collected for perchlorate, dissolved oxygen, and

nitrate, perchlorate removal was 90% for F120 (15-min EBCT) but only 63% for F130 (18-min EBCT). At the time of biomass sampling, F120 was operated with a 10-min EBCT, and F130 was operated with a 15-min EBCT.

The *cld* and *pcrA* qPCR assays were used to quantify gene concentrations with depth in the bioreactors. For both F120 and F130, *pcrA* and *cld* gene copy numbers were highest at the top of the bed (Figure 4) where perchlorate removal was the highest. However, *pcrA* copies were two- to three-fold higher, and *cld* copies were 3- to 4-fold higher throughout the fixed-bed in F120 as compared to F130. This suggests that a greater fraction of the microbial community was PRB in F120, which might account for the superior perchlorate removal performance (assuming that both bioreactors had similar amounts of total biomass). Similar to the observations in section 4.2 for the bench-scale bioreactor, the quantity of *pcrA* genes detected was approximately one order of magnitude lower than the quantity of *cld* genes detected in the pilot-scale bioreactors, suggesting imperfections in the existing *pcrA* qPCR assay.

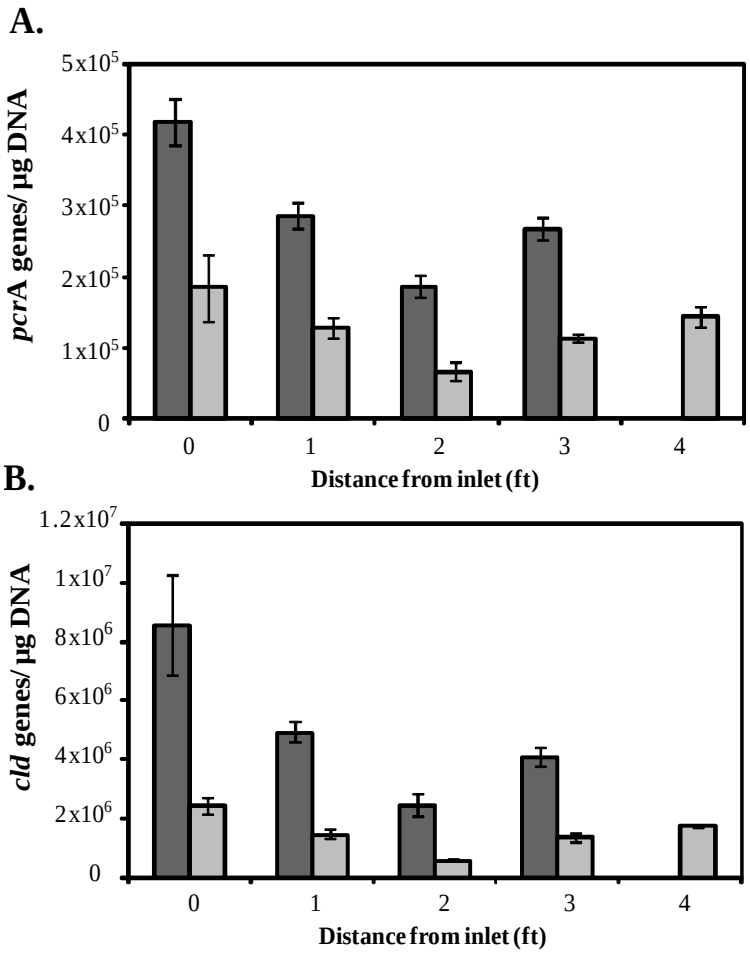

Figure 4. *pcrA* and *cld* gene concentrations as a function of depth in the pilot-scale bioreactors. A. *pcrA* and B. *cld* gene concentrations in reactors F120 and F130 (dark gray bars and light gray bars, respectively) [Copyright © John Wiley & Sons, Inc., De Long *et al.*, *J Appl Microbiol.* 112(3):579-592, 2012]

4.4 Results from Task 9 (Address potential bias in the quantitative assay for *pcrA*)

The purpose of this task was to test the potential bias of the existing *pcrA* qPCR assay (Nozawa-Inoue *et al.* 2008). Our previous work showed that that assay provides accurate *pcrA* quantification for *D. aromatica* but appears to provide inaccurate *pcrA* quantification for *D. agitata*. De Long *et al.* (2010) reported the total number of primer mismatches (forward plus reverse primer) for those isolates: four mismatches each with the *pcrA* sequence in *D. agitata* and the sequence of *D. aromatica*. Although the number of mismatches for the two isolates is the same, the positions of the mismatches are different. For instance, in the forward primers, the first mismatch location for *D. aromatica* is in the second position from the 5' end of the primer but that for *D. agitata* is in the fifth position from the 5' end of the primer. The effects of internal primer-template mismatches on the efficiency of PCR amplification using the 16S rRNA gene have been reported (Bru *et al.* 2008). They concluded that the mismatch position of the primer used could bias qPCR assays by leading to an underestimation of the actual gene concentration.

We designed new *pcrA* primer sets to be perfect matches to several perchlorate-reducing strains: *Dechloromonas* sp. PC1, *Dechloromonas aromatica* RBC, *Dechloromonas agitata* CKB, and *Azospira* sp. KJ (Table 5).

Standard curves were generated with the *pcrA* primers using *D. aromatica* genomic DNA (Figure 5). Primer sets pcrA0 (pcrA320F/598R) and pcrA2 (perfect match to *pcrA* sequence in *D. aromatica* RCB) successfully amplified genomic DNA from *D. aromatica*, but the pcrA0 primer set showed lower yield (i.e., for a given copy number, it took a greater number of cycles to reach the threshold concentration with primer set pcrA0 as compared to perfectly matched primer set pcrA2). However, primer sets pcrA1, pcrA3, pcrA4, and pcrA5 did not amplify *D. aromatica* genomic DNA well, indicating that a higher number of mismatches between the primers and the template caused substantially lower yield.

Nozawa Inoue *et al.* (2008) cloned the *pcrA* gene from *D. agitata* CKB into a plasmid using *pcrA* that had been amplified with primer set pcrA0. Standard curves also were generated with the *pcrA* primers using this plasmid DNA (Figure 6). For this DNA, the pcrA0, pcrA1, pcrA2, pcrA4, and pcrA5 primer sets showed linear standard curves, but the pcrA0 primer set showed the highest yield because the cloned gene had previously been amplified with pcrA0.

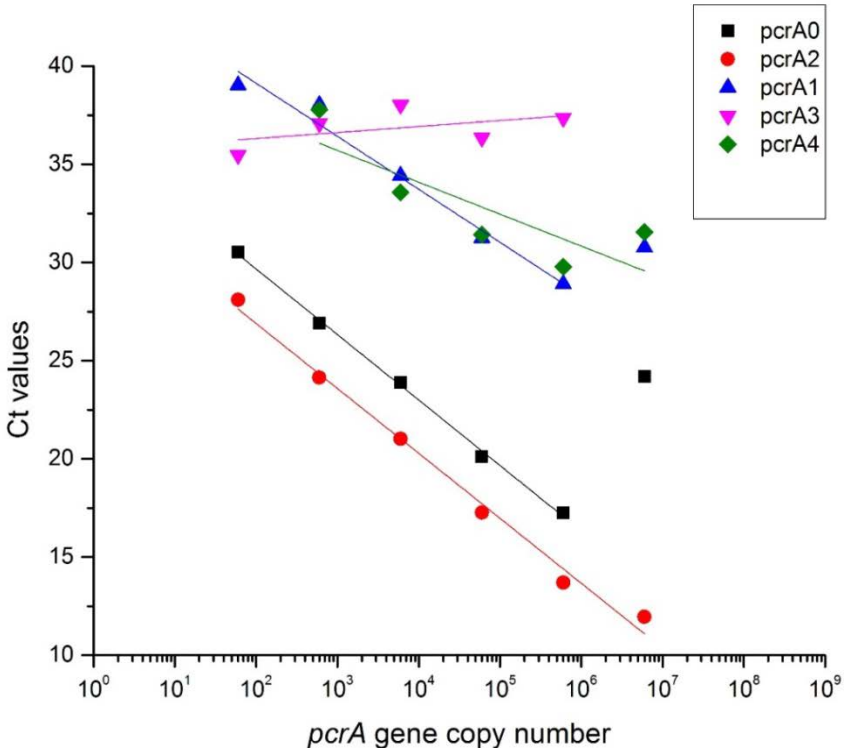

Figure 5. *pcrA* standard curves using *D. aromatica* DNA with perfectly matched (pcrA2) or mismatched primer sets

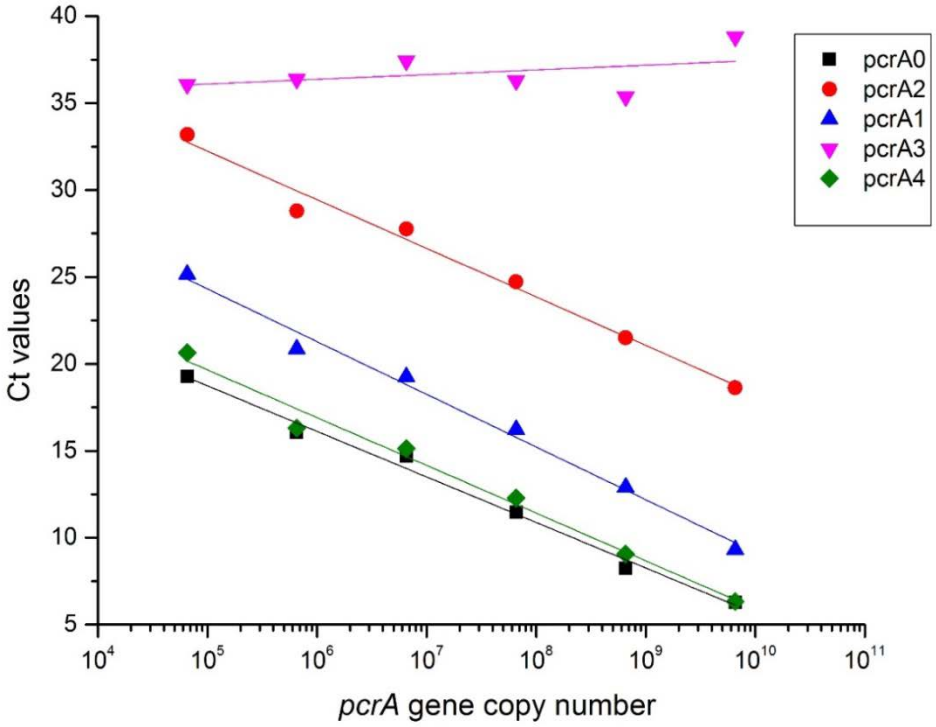

Figure 6. *pcrA* standard curves using *pcrA* from *D. agitata* on a plasmid with perfectly matched (pcrA0) or mismatched primer sets

The *pcrA* gene concentration was measured in PRB with the primer sets that were a perfect match to the target strains. For the strains listed in Table 9, the estimated number of gene copies spiked to the qPCR reactions ($5x10^6$) was calculated based on an assumed bacterial genome size of 4501 kb (which is the *D. aromatica* genome size) and an assumed one copy of *pcrA* per bacterial genome (which is the number of *pcrA* copies in the *D. aromatica* genome). The measured gene copy numbers were calculated using the standard curve based on the pcrA0 primer set with plasmid DNA containing the cloned *pcrA* gene from *D. agitata* CKB that had been amplified with primer set pcrA0 (Figure 6), and average measured values are reported in Table 9.

Table 9. Comparison of the measured number of *pcrA* gene copies and the spiked number of gene copies (Approximate spike of $5x10^6$ gene copies/reaction)

Strains	pcrA0	pcrA1	pcrA2	pcrA3	pcrA4
D. agitata CKB	9×10^4	**6.2×10^6**	7.6×10^0	1.3×10^3	-
D. aromatica RCB	6×10^5	-	**4.1×10^6**	6.1×10^4	1.2×10^2
Azospira sp. KJ	6.8×10^1	-	7.6×10^2	**8.6×10^6**	8.8×10^1
Dechloromonas sp. PC1	5.1×10^2	3.85×10^0	2.3×10^2	3.4×10^2	**7.6×10^6**
Rhodocyclaceae strain JDS4	2.6×10^3	-	1.8×10^2	1.1×10^2	3.5×10^1

**Yellow cells indicate a perfect match between primer set and *pcrA* gene of that bacterium

Consistent with our expectations, the measured number of *pcrA* gene copies was close to the spiked number of *pcrA* gene copies (5×10^6) in qPCR reactions with *D. aromatica* DNA and primer set pcrA0; additionally, the measured *pcrA* gene copy number was less than the spiked *pcrA* gene copy number (9×10^4 instead of 5×10^6) in qPCR reactions with *D. agitata* genomic DNA and primer set pcrA0 (Table 9). The measured number of gene copies in qPCR reactions with *D. aromatica*, *D. agitata*, *Azospira* sp. KJ or *Dechloromonas* sp. PC1 and their respective perfectly matched primers sets were on the same order as the spiked number of gene copies ($5x10^6$). However, when *D. agitata* and *D. aromatica* were tested with primer sets containing mismatches to their *pcrA* genes, the measured number of gene copies was less than the spiked number of gene copies, often by one or two orders of magnitude. Therefore, mismatches between the primers and template resulted in substantial undercounts of the number of *pcrA* gene copies. Although the *pcrA* genes of *D. aromatica* and *D. agitata* each have four total mismatches with primer set pcrA0, *D. aromatica* showed better amplification with that primer set than did *D. agitata* ($6x10^5$ and $9x10^4$, respectively). Thus, the position of the mismatches also might cause substantial undercounts of the *pcrA* gene copy number.

Interestingly, DNA from JDS4 did not show strong amplification with any of the tested *pcrA* primer sets (Table 9), which suggests sequence variation in *pcrA*.

In summary, this task demonstrated the bias of the existing *pcrA* qPCR assay, where mismatches between the template and primer resulted in substantially lower measured numbers of gene copies.

4.5 Results from Task 10 (Increase rRNA removal in the Prokaryotic cDNA Subtraction protocol)

Using *Rhodocyclaceae* strain JDS4, the minimum amount of total RNA recommended (2 µg) was obtained using the Ribopure™-Bacteria Kit (Ambion, Austin, TX), and then mRNA was purified with the MICROBexpress™ kit (Table 10). As expected, mRNA quantities decreased after each round of mRNA purification (Table 10).

Table 10. Quantities of total RNA and mRNA after various purification steps

	RNA mass (µg)			
Strain	Total RNA	mRNA (after 1st round MICROBexpress™)	mRNA (after 2nd round MICROB*express*™)	mRNA (after 3rd round MICROBexpress™)
JDS4	5.0	1.0	0.7	0.4

After the various rounds of mRNA purification, the RNA was used to generate cDNA, and the quantities of cDNA produced from the 16S and 23S rRNA remaining in the purified mRNA was examined (Table 11). The quantities of 16S rRNA and 23S rRNA decreased after each round of mRNA purification (i.e., C_t increased), but the overall removal of 23S rRNA was less than that for 16S rRNA (96.48% and 99.90% after three rounds of purification, respectively). The 23S rRNA was substantially removed from total RNA after round 2 and round 3 of mRNA purification with the MICROBexpress™ kit. If the ΔC_t values were three between two samples, the number of gene copies theoretically differs by one order of magnitude. For example, the 23S rRNA copy number decreased by one to two orders of magnitude for each round of mRNA purification (Table 11). Residual 16S and 23S rRNA were still detectable after three rounds of mRNA purification (i.e., present in greater quantities than the negative control with no added template).

Table 11. Average C_t values and percent removal of rRNA after mRNA purification from total RNA of strain JDS4

rRNA	Cumulative percent rRNA removal and C_t value				C_t value negative control
	After 2nd round MICROBexpress™	C_t value	After 3rd round MICROBexpress™	C_t value	
16S rRNA	98.73%	31.5	99.90%	33.5	Undetermined
23S rRNA	87.95%	26	96.48%	31.5	38

Due to residual 16S and 23S rRNA in the purified mRNA, the complementarity between the rRNA sequences of JDS4 and the capture oligonucleotides that are supposed to bind rRNA in the MICROBexpress™ kit was investigated. The sequences of 16S and 23S rRNA were submitted to Ambion (manufacturer of the MICROBexpress™ kit), who determined that the capture oligonucleotides in the MICROBexpress™ kit were a perfect match to the 16S rRNA of JDS4 but an imperfect match to the 23S rRNA of JDS4, resulting in reduced 23S rRNA removal (Table 11). Since customized capture oligonucleotides were unavailable for the MICROBexpress™ kit, we tested a new mRNA purification kit: Ribo-Zero™ rRNA removal kit (Epicentre® Madison, WI), whose capture oligonucleotides are perfectly complementary to the

16S and 23S rRNA of JDS4. The removal of 16S rRNA and 23S rRNA from total RNA extracted from JDS4 was examined with the Ribo-Zero™ rRNA removal kit alone or in combination with the MICROBexpress™ kit. As shown in Table 12, the removal of 16S rRNA and 23S rRNA was increased by using the combination of the two rRNA removal kits (MICROBexpress™ and Ribo-Zero™ rRNA removal kits).

Table 12. Cumulative percent removal of 16S and 23S rRNA via mRNA purification

Protocols	Percent Removal (%)	
	16S rRNA	23S rRNA
Ribo-Zero™ rRNA removal kit	98.97	99.41
MICROBexpress™ kit + Ribo-Zero™ rRNA removal kit	99.95	99.99

In summary, the sequential purification of mRNA from total RNA with two kits (Ribo-Zero™ rRNA removal kit and MICROBexpress™ kit) is recommended for use in the Prokaryotic cDNA Subtraction protocol. As in the case with strain JDS4, differences in the capture oligonucleotide sequences between the two kits can allow for improved rRNA removal as compared to the use of a single kit.

4.6 Results from Task 11 (Modify the hybridization step in the Prokaryotic cDNA Subtraction protocol)

The purpose of this task was to test the Prokaryotic cDNA Subtraction protocol with defined tester and driver pools, where the driver pool contained purposefully elevated numbers of 16S/23S copies. Purposefully elevated 16S/23S copies in the driver would increase the driving force for their hybridization to their complements in the tester, thereby preventing these molecules from being amplified in suppression PCR; this would increase the probability with which biodegradation genes are identified in the Prokaryotic cDNA Subtraction.

Defined tester and driver pools containing DNA copies of only 16S rRNA, 23S rRNA, *cld*, and *pcrA* were constructed (Table 6). As shown in Figure 7, the constructed driver pool was designed to contain high concentrations of DNA copies of 16S and 23S rRNA (i.e., visible in the gel, Lane 1). The constructed tester pool contained high concentrations of DNA copies of *cld* and *pcrA*, with low concentrations of DNA copies of 16S and 23S rRNA (Figure 7, Lane 2); this scenario is expected in a real subtraction experiment, where the functional transcripts of interest (i.e., *cld* and *pcrA*) would be elevated in the tester culture (i.e., grown under perchlorate-reducing conditions) as compared to the driver culture, and rRNA would be low due to mRNA purification.

Prokaryotic cDNA Subtraction was performed on the constructed tester and driver. Tester DNA was ligated to adaptors 1 and 2 and then hybridized with driver DNA twice as shown as Figure 1. The subtraction successfully increased the *cld* and *pcrA* concentrations relative to the 16S and 23S rRNA gene concentrations in the tester DNA (Figure 7, Lane 3).

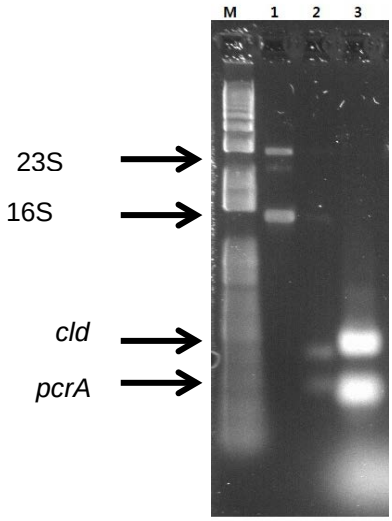

Figure 7. Results of the Prokaryotic cDNA Subtraction on the constructed tester and driver pools M: marker. Lane 1: the driver pool, Lane 2: the tester pool, Lane 3: Forward-subtraction with constructed tester and driver pools

In summary, having higher 16S and 23S rRNA in the driver as compared to the tester might increase subtraction efficiency, improving the probability of identifying functional genes not related to rRNA in the Prokaryotic cDNA Subtraction. In situations where mRNA purification is inadequate to increase the frequency of functional gene identification to acceptable levels in the Prokaryotic cDNA Subtraction, the hybridization step might be modified as in this task to allow for higher concentrations of 16S and 23S rRNA in the driver pool.

4.7 Results from Task 12 (Deploy modified Prokaryotic cDNA Subtraction protocol on PRB strain JDS4)

The purpose of this task was to deploy the modified Prokaryotic cDNA Subtraction protocol on the environmental perchlorate-reducing isolate JDS4.

First, JDS4 was grown in batch cultures under anaerobic conditions; then, half of the cultures were maintained under perchlorate-reducing conditions (tester culture) and half were converted to aerobic conditions (driver culture). Total RNA was extracted from both cultures, and mRNA was purified with the MICROBexpress™ Bacterial mRNA Enrichment Kit followed by the Ribo-Zero™ rRNA removal kit (Task 10). Greater than 99.0% removal of 16S and 23S rRNA from the total RNA was obtained (Table 13).

Table13. Percent removal of 16S and 23S rRNA after mRNA purification

Target	Aerobic condition (without perchlorate=driver)	Anaerobic condition (with perchlorate=tester)
16S rRNA	99.97%	99.97%
23S rRNA	99.87%	99.80%

The Prokaryotic cDNA Subtraction using this purified mRNA was carried out as described in De Long *et al.* (2008). Prokaryotic cDNA Subtraction should identify genes that are up-regulated under perchlorate-reducing conditions as compared to aerobic conditions. Pyrosequencing was used to identify those up-regulated genes using the MG-RAST pipeline (Meyer *et al.* 2008). MG-RAST analysis included 9,708 sequences totaling 3,297,139 bps with an average length of 339 bps. A small number of sequences (1.8%) failed to pass the QC in the MG-RAST pipeline and were discarded. Of the sequences that passed QC, 4,172 sequences (43.0%) are from rRNA and 5,359 sequences (55.2%) have other functions (i.e., are not rRNA). Thus, the amended mRNA purification improved rRNA removal, but it did not completely eliminate rRNA. However, pyrosequencing allowed us to identify many functional sequences unrelated to rRNA including three related to perchlorate reduction: perchlorate reductase alpha subunit precursor, perchlorate reductase A, and perchlorate reductase B (Table 14). A comparison of the recovered sequences to those in Genbank indicates that the gene sequences encoding the perchlorate reductase alpha subunit precursor of JDS4 were close to those found in *Dechloromonas agitata*, and the gene sequences encoding perchlorate reductase A and B were similar to those found in *Azospira* sp. KJ.

Table 14. Functional genes related to perchlorate reduction that were detected in a Prokaryotic cDNA Subtraction experiment with strain JDS4

Source	Enzyme subunit	Abundance[a]	Log E value[b]	Avg % identity	Source of GenBank[c] (Accession number)
GenBank	perchlorate reductase alpha subunit precursor	6	-17.75	89.7	*D. agitata* AAO49008.1
	perchlorate reductase A	2	-15	88.9	*Azospira* sp. KJ ACB69917.1
	perchlorate reductase B	1	-34	86.7	*Azospira* sp. KJ ACB69918.1

[a] Abundance describes the number of times the functional role was detected in the sequences
[b] E value (expected value) is an indicator for estimating sequence similarity.

The *pcrA* sequence recovered from the Prokaryotic cDNA Subtraction experiment with strain JDS4 was compared with the existing *pcrA* primers (Nozawa-Inoue *et al.* 2008). The region complementary to pcrA320F was found, but there were seven mismatches between the primer and the *pcrA* sequence in JDS4. This explains the low amplification of *pcrA* in JDS4 with the existing *pcrA* primers (Table 9).

In summary, Prokaryotic cDNA Subtraction was successfully applied to the PRB JDS4 via improvements in the protocol (i.e., increased rRNA removal and the use of pyrosequencing). Prokaryotic cDNA Subtraction identified perchlorate reductase genes as being up-regulated under perchlorate-reducing conditions, thereby allowing the expansion of the functional gene database for perchlorate reduction.

5.0 Conclusions and Implications for Future Research

Perchlorate is a stable, soluble contaminant that occurs in the environment due to anthropogenic and natural activities. Some microorganisms can reduce perchlorate to innocuous chloride, and that potential can be harnessed for the biological treatment of perchlorate-contaminated waters. ERSON-07-03 affirmed the need for methods to identify biomarkers associated with the biodegradation of compounds relevant to the DoD, such as perchlorate. Related to this need, the purpose of the current project was to improve our ability to detect the potential for biological perchlorate reduction at a contaminated site and to monitor the transcriptional activity of PRB in a treatment system. Specific objectives for the project were (1) to refine quantitative assays for genes associated with microbial perchlorate reduction and to apply quantitative assays on bench-scale and pilot-scale perchlorate-reducing bioreactors; and (2) to improve the protocol for the focused gene discovery technique Prokaryotic cDNA Subtraction so that the database of perchlorate-related gene sequences could be expanded.

One key enzyme in the perchlorate reduction pathway is chlorite dismutase, which is encoded by the *cld* gene. A qPCR assay for *cld* has been published (De Long *et al.* 2010), but improvements were needed to improve amplification efficiency in the *cld* assay. We redesigned the *cld* qPCR forward primer to decrease the amplicon size from ~400 bps to ~200 bps, which is in the optimal size range for qPCR. The primer re-design coupled with changes in the thermal cycler profile resulted in substantial increases in PCR amplification efficiency and improved detection of lower *cld* copy numbers, thus addressing a practical gap in knowledge.

Another key enzyme in the perchlorate reduction pathway is perchlorate reductase, which is encoded by the *pcrABCD* genes. A qPCR assay for *pcrA* has been published (Nozawa-Inoue *et al.* 2008), but our previous work showed that while the assay accurately quantifies *pcrA* for *D. aromatica* it inaccurately quantifies *pcrA* for *D. agitata*. Although the total number of primer mismatches with *pcrA* in *D. aromatica* and *D. agitata* is the same (four), the positions of the mismatches are different. Thus, the mismatch position appears to impact *pcrA* quantification. Primer-template mismatches at the 3' end of the primer have traditionally been thought to be more important because the 3' end is where primer extension occurs. However, the mismatches between the *pcrA* primers and templates tested in this project were concentrated at the 5' end of the primer, and the *pcrA* quantities measured were often one or more orders of magnitude below the spiked concentration. We designed new *pcrA* primer sets to be perfect matches to several perchlorate-reducing strains: *Dechloromonas* sp. PC1, *Dechloromonas aromatica* RBC, *Dechloromonas agitata* CKB, and *Azospira* sp. KJ. Each primer set amplified *pcrA* in its target strain well, but did not amplify *pcrA* from the other tested PRB. These primer sets might be used in parallel to more accurately quantify *pcrA* from PRB in a mixed microbial community, thus addressing a gap in the ability of the scientific community to monitor PRB in field systems.

The existing *cld* (De Long *et al.* 2010) and *pcrA* (Nozawa-Inoue *et al.* 2008) qPCR assays were used to quantify gene or transcript concentrations in bench-scale and pilot-scale perchlorate-reducing bioreactors. At the bench-scale, the concentration of *cld* transcripts was two- to eight-fold lower under near-failure conditions (<25% perchlorate removal) than during normal operation (>50% perchlorate removal), which suggests that the near-failure in the bioreactor might have been due in part to a loss of PRB from the reactor. At the pilot-scale, *cld*

concentrations were three- to four-fold higher in the fixed-bed reactor that showed 90% perchlorate removal as compared to the fixed-bed reactor that showed 63% perchlorate removal, which suggests that a larger PRB community was present in the reactor that demonstrated superior perchlorate removal. Thus, it was concluded that the concentrations of *cld* genes and transcripts are likely to be a good indicator of bioreactor health, thus addressing a gap in understanding regarding the applicability of molecular methods to monitor treatment system health for perchlorate reduction.

The bench-scale and pilot-scale data both underscored the potential for the existing *pcrA* qPCR to undercount *pcrA*. In the bench-scale bioreactor, the quantity of *pcrA* transcripts detected was two to three orders of magnitude lower than the quantity of *cld* transcripts detected. Similarly, in the pilot-scale bioreactors, the quantity of *pcrA* genes detected was approximately one order of magnitude lower than the quantity of *cld* genes detected. To fully interrogate bioreactor health with respect to perchlorate removal, accurate *pcrA* quantification is required; *cld* quantification alone is insufficient because some microorganisms have *cld* but not *pcrA* and are therefore unable to reduce perchlorate. Thus, these data underscored the lack of qPCR assays to accurately quantify *pcrA* in mixed microbial communities.

Since few perchlorate-related gene sequences are known, primers and probes for interrogating the potential for perchlorate degradation at a contaminated site or the transcriptional activity of PRB in a treatment system have been narrowly designed. Prokaryotic cDNA Subtraction (De Long *et al.* 2008) is a focused gene discovery technique that can be used to identify genes that are up-regulated under one set of conditions relative to another (e.g., perchlorate-reducing conditions in the "tester" culture as compared to aerobic conditions in the "driver" culture). Thus, this technique could be useful for expanding the database of gene sequences related to perchlorate degradation such that better biomarkers could be designed. A key part of the Prokaryotic cDNA Subtraction method is to purify high quality mRNA under the comparative conditions. However, when we first applied the Prokaryotic cDNA Subtraction technique to a PRB in our previous project (Kirisits *et al.* 2008), residual rRNA in the mRNA prevented the identification of perchlorate-related genes in that PRB; instead 29 out of the 30 sequences (97%) identified were 16S rRNA or 23S rRNA. To rectify this situation, two modifications to the Prokaryotic cDNA Subtraction protocol were evaluated in the current project for application to PRB strain JDS4 in the *Rhodocyclaceae* family. First, rRNA removal from mRNA was improved through the sequential application of two different purification kits that had different capture oligonucleotides for rRNA. Second, purposefully increasing the rRNA residual in the driver as compared to the tester mRNA was demonstrated to be a potential way to decrease the identification of rRNA in the sequences from Prokaryotic cDNA Subtraction. The first modification (sequential mRNA purification with two different kits) was deployed in a Prokaryotic cDNA Subtraction on strain JDS4 where the final sequences were obtained through pyrosequencing. The modified protocol was highly successful, where 55% of the genes identified were functional genes unrelated to rRNA. Three perchlorate-related genes were identified, encoding the perchlorate reductase alpha subunit precursor, perchlorate reductase A, and perchlorate reductase B. The *pcrA* sequence of JDS4 had seven mismatches with the existing forward *pcrA* primer (Nozawa-Inoue *et al.* 2008), which explains its poor amplification with that primer set. Thus, we have taken an important step in addressing a knowledge gap: the development of an expanded functional gene database related to perchlorate reduction.

In summary, the objectives for the project were met. The utility of qPCR or RT-qPCR *cld* assays for monitoring the health of perchlorate-reducing bioreactors was demonstrated at the bench- and pilot-scale; furthermore, the undercounting of *pcrA* copies with the existing *pcrA* qPCR assay was demonstrated, highlighting the need to expand the functional gene database for more inclusive *pcrA* primer design. The qPCR assay for *cld* was refined to increase the amplification efficiency and improve its detection of low *cld* copies, and additional *pcrA* primer sets were developed to quantify *pcrA* genes not captured in the existing *pcrA* primer set. **These refined qPCR and RT-qPCR assays can now be directly applied to monitor the potential for biological perchlorate reduction at a contaminated site or the transcriptional activity of PRB in a treatment system; thus, these assays are useful at DoD sites and drinking-water treatment plants practicing biological perchlorate reduction.** Additionally, the Prokaryotic cDNA Subtraction protocol was improved and successfully deployed to strain JDS4 in the *Rhodocyclaceae* family, an environmental perchlorate-reducing isolate. This exciting development resulted in the identification of a *pcrA* sequence in JDS4 that has substantial differences from other known *pcrA* sequences, which means that we have taken the first, important step in expanding the functional gene database related to perchlorate reduction. **The improved Prokaryotic cDNA Subtraction protocol can now be applied to other PRB as well as to other prokaryotes that transform contaminants of interest to the DoD, such as 1,4-dioxane, 2,4,6-trinitrotoluene (TNT), 2,4-dinitrotoluene (DNT), and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), to develop new or improved biomarkers.**

Building on the success of this project, several fruitful research avenues could be pursued in a subsequent study. First, a multiplex Taqman® assay using a degenerate primer set could be designed to collapse the multiple *pcrA* primers developed for improved PRB quantification to a single assay. Second, additional PRB could be interrogated via Prokaryotic cDNA Subtraction to continue to expand the functional gene sequence database for perchlorate reduction. Third, qPCR assays for *cld* and *pcrA* could be applied to monitor the health of a full-scale biological drinking water treatment facility over time. Dr. Todd Webster (Envirogen Technologies, Inc.) has agreed to provide samples from the full-scale fluidized-bed reactor that his firm designed for biological perchlorate reduction in drinking water (Rialto, California). Fourth, our anaerobic membrane bioreactor for perchlorate reduction could be interrogated with the *cld* and *pcrA* qPCR assays before and after perturbation to determine the resiliency of PRB under stress.

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Appendix 1: List of Scientific/Technical Publications

Articles in peer-reviewed journals (in print)

De Long, S. K., K. A. Kinney, and M. J. Kirisits. (2010) "qPCR Assays to Quantify Genes and Gene Expression Associated with Microbial Perchlorate Reduction." *Journal of Microbiological Methods* 83:2:270-274.

De Long, S. K., X. Li, S. Bae, J. C. Brown, L. Raskin, K. A. Kinney, and M. J. Kirisits. (2012) "Quantification of Genes and Gene Transcripts for Microbial Perchlorate Reduction in Fixed-Bed Bioreactors." *Journal of Applied Microbiology* 112:3:579-592.

Conference abstracts

Bae, S., K. A. Kinney, and M. J. Kirisits. (2010) "Quantifying *cld* and *pcrA* Genes from Intact Perchlorate-reducing Cells as Measured by PMA-qPCR." *Abstracts of the Partners in Environmental Technology Technical Symposium and Workshop*. Washington, D.C.