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Impact of Fixed Nitrogen Availability on *Dehalococcoides mccartyi* Reductive Dechlorination Activity

Devrim Kaya,[†,‡,&,#] Birthe V. Kjellerup,[#] Karuna Chourey,[⊥,○] Robert L. Hettich,[⊥] Dora M. Taggart,[▽] and Frank E. Löffler*[,†,‡,§,∥,&]

[†]Center for Environmental Biotechnology, [‡]Department of Microbiology, [§]Department of Civil and Environmental Engineering, and [∥]Department of Biosystems Engineering & Soil Science, University of Tennessee, 676 Dabney Hall, 1416 Circle Drive, Knoxville, Tennessee 37996, United States

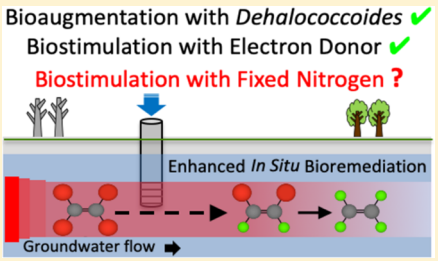
[&]Biosciences Division and [⊥]Chemical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, United States

[#]Department of Civil and Environmental Engineering, University of Maryland College Park, College Park, Maryland 20742, United States

[▽]Microbial Insights, Inc., Knoxville, Tennessee 37932, United States

Supporting Information

ABSTRACT: Biostimulation to promote reductive dechlorination is widely practiced, but the value of adding an exogenous nitrogen (N) source (e.g., NH_4^+) during treatment is unclear. This study investigates the effect of NH_4^+ availability on organohalide-respiring *Dehalococcoides mccartyi* (*Dhc*) growth and reductive dechlorination in enrichment cultures derived from groundwater (PW4) and river sediment (TC) impacted with chlorinated ethenes. In PW4 cultures, the addition of NH_4^+ increased *cis*-1,2-dichloroethene (cDCE)-to-ethene dechlorination rates about 5-fold (20.6 ± 1.6 versus 3.8 ± 0.5 μM Cl^- d^{-1}), and the total number of *Dhc* 16S rRNA gene copies were about 43-fold higher in incubations with NH_4^+ ((1.8 ± 0.9) × 10^8 mL^{-1}) compared to incubations without NH_4^+ ((4.1 ± 0.8) × 10^7 mL^{-1}). In TC cultures, NH_4^+ also stimulated cDCE-to-ethene dechlorination and *Dhc* growth. Quantitative polymerase chain reaction (qPCR) revealed that Cornell-type *Dhc* capable of N_2 fixation dominated PW4 cultures without NH_4^+, but their relative abundance decreased in cultures with NH_4^+ amendment (i.e., 99 versus 54% of total *Dhc*). Pinellas-type *Dhc* incapable of N_2 fixation were responsible for cDCE dechlorination in TC cultures, and diazotrophic community members met their fixed N requirement in the medium without NH_4^+. Responses to NH_4^+ were apparent at the community level, and N_2-fixing bacterial populations increased in incubations without NH_4^+. Quantitative assessment of *Dhc* nitrogenase genes, transcripts, and proteomics data linked Cornell-type *Dhc nifD* and *nifK* expression with fixed N limitation. NH_4^+ additions also demonstrated positive effects on *Dhc* in situ dechlorination activity in the vicinity of well PW4. These findings demonstrate that biostimulation with NH_4^+ can enhance *Dhc* reductive dechlorination rates; however, a "do nothing" approach that relies on indigenous diazotrophs can achieve similar dechlorination end points and avoids the potential for stalled dechlorination due to inhibitory levels of NH_4^+ or transformation products (i.e., nitrous oxide).



INTRODUCTION

Groundwater aquifers are often oligotrophic and cannot sustain high-rate reductive dechlorination desirable at sites contaminated with chlorinated solvents.[1−3] Enhanced anaerobic bioremediation at sites impacted with chlorinated ethenes relies on biostimulation with fermentable substrates to increase hydrogen flux.[3−6] Hydrogen is the key electron donor for organohalide-respiring *Dehalococcoides mccartyi* (*Dhc*) strains capable of dechlorination to environmentally benign ethene.[7] In situ growth of *Dhc* in response to biostimulation with fermentable substrates has been documented;[8−11] however, a decline in dechlorination rates and incomplete reductive dechlorination at sites that receive sufficient electron donor is a common challenge to meet remedial goals.[12,13] While hydrogen and chlorinated ethenes meet *Dhc*'s energy requirement and acetate generated in fermentation reactions serves as a carbon source, fixed nitrogen (N) availability may limit *Dhc* growth and reductive dechlorination activity.[14]

Ubiquitous dinitrogen (N_2) must be reduced to ammonium (NH_4^+) to serve anabolic purposes; however, N_2 fixation is an energetically expensive process (16 ATP consumed per N_2 molecule reduced to NH_4^+) and only occurs when NH_4^+ is limiting.[15] The nitrogenase enzyme complex Nif, encoded by *nifH, nifD*, and *nifK* (*nif* operon), catalyzes the reduction of N_2 to NH_4^+.[16] The *nifH* gene has been used as a biomarker for

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Table 1. New Primers and Probes Designed in this Study for qPCR and Reverse Transcriptase (RT)-qPCR Analyses[a]

Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	TaqMan-MGB probe (5′ → 3′)	Target gene (organism)
Dhc Cornell 16S-qF: AACTGAAGGTAATACCGCATGTGAT	*Dhc* Cornell 16S-qR: ATCCTCTCAGACCAGCTACCGA	*Dhc* Cornell 16S-qP: TAAGTCGGTTCATTAAAGC	16S rRNA (Cornell-type *Dhc*)
nifD-195-650qF: ATAACCCGTTCGGTCTGTTC	*nifD*-195-769qR: TACGGCATTCCCTGGATTAAG	*nifD*-195-687P: CGAAATAACGGGCAATCTTGCGCA	*nifD* (Cornell-type *Dhc*)
nifK-195-37qF: ATCATTTCCAGCAAACGCATT	*nifK*-195-119qR: GGCTTCCCCATTCTTGACC	*nifK*-195-64qP: CGATAGCCCACAACC	*nifK* (Cornell-type *Dhc*)
nifH-195C-69qF: GCCCGGTATTCGTCAGCC	*nifH*-195C-147qR: ATGTCCAGCGGGCTGAAAT	*nifH*-195C-96qP: TGCGGATTCCATTCG	*nifH* (Cornell-type *Dhc*)
nifH-PV-362qF AGCATGCCAGATTCATTACCG	*nifH*-PV-466qR: GGCTGCAACTGAACATAGGTCC	*nifH*-PV-400qP: TCCAAGACTATGGGTGTGAC	*nifH* (Pinellas- and Victoria-type *Dhc*)

[a]Primer/probe sets *nifH-195C* and *nifH-PV* were designed to target the *nifH* gene of the N_2-fixing Cornell-type *Dhc* and the non-N_2-fixing Pinellas- and Victoria-type *Dhc*, respectively, while primer/probe sets for *Dhc* Cornell 16S rRNA genes were designed to distinguish N_2-fixing *Dhc* (i.e., Cornell group) from the non-N_2-fixing *Dhc* (Pinellas and Victoria groups). Primer/probe design used the Primer3 software (Applied Biosystems, Foster City, CA).[68] Abbreviations: q: qPCR, F: forward primer, R: reverse primer, P: probe, *Dhc*: *Dehalococcoides mccartyi*, MGB: minor groove binder.

N_2-fixing microorganisms in ecological and phylogenetic studies;[17] however, the expression of *nifH* alone does not predict N_2 fixation activity.[18] The expression of the entire *nif* operon underlies stringent regulation, and *nifHDK* transcripts serve as indicators for N_2 fixation activity and fixed N limiting conditions.[19]

Dhc are key players for detoxification of chlorinated ethenes to environmentally benign ethene.[7,20] Based on 16S rRNA gene sequence differences, the *Dhc* genus is divided into the Cornell, the Victoria, and the Pinellas groups (Figure S1).[21] The presence of complete and functional *nif* operons in the genomes of Cornell-type *Dhc* strains indicates that N_2 fixation is a shared trait of strains belonging to this group.[14,22−24] In contrast, the Pinellas and Victoria subgroups have incomplete *nif* operons and cannot fix N_2.[25]

Little is known about the consequences of NH_4^+ limitation and the efficacy of NH_4^+ biostimulation on *Dhc* growth and reductive dechlorination activity. This study explored the impact of NH_4^+ on reductive dechlorination using two *cis*-1,2-dichloroethene- (cDCE-) to-ethene-dechlorinating enrichment cultures derived from sites impacted with chlorinated solvents: the river sediment-derived TC enrichment with Pinellas/Victoria group *Dhc* but lacking Cornell group *Dhc* and the groundwater aquifer-derived PW4 enrichment culture dominated by Cornell group *Dhc*. Furthermore, we analyzed samples collected from monitoring well PW4 to investigate the effect of fixed N biostimulation at a groundwater aquifer impacted with chlorinated ethenes.

MATERIALS AND METHODS

Chemicals. cDCE, vinyl chloride (VC), and ethene (greater than 99% purity) were obtained from Sigma-Aldrich-Fluka (St. Louis, MO). Formic acid (99%) was purchased from Fisher Scientific (Waltham, MA), and sequencing-grade trypsin was acquired from Promega (Madison, WI).

Cultures and Growth Conditions. Groundwater samples were collected in January 2014 from well PW4 located at a contaminated site in Australia and shipped on ice for immediate processing upon arrival.[10] PW4 enrichment cultures were established in 160 mL serum bottles by combining 50 mL of site groundwater with 50 mL of defined mineral salts medium[26] with the following modifications: dithiothreitol (DTT) (0.5 mM) replaced L-cysteine as a reducing agent to eliminate a possible N source. After complete dechlorination of cDCE to ethene, transfers (2%, v/v) were carried out in larger vessels (2 L glass bottles containing 1.7 L of the medium) to accommodate larger sample requirements for proteomic analysis. TC enrichment cultures were derived from Third Creek sediment impacted with the chlorinated solvent following established procedures.[27] Neat cDCE (approximately 0.3 mM aqueous phase concentrations) as the electron acceptor and lactate (5 mM), a fermentable substrate yielding hydrogen as the electron donor for *Dhc*, were added to TC and PW4 incubation vessels. To assess the effect of NH_4^+, cultures were established with NH_4^+ (5.6 mM or 0.08 g L^{-1}) amendment or without NH_4^+ (Table S1). The headspace consisted of N_2/CO_2 (80/20, v/v), except for negative controls of N_2 fixation activity, where argon replaced N_2 and NH_4^+ was omitted from the medium (Table S1). *Dhc* strains BAV1 and GT were grown in 160 mL serum bottles containing 100 mL of defined medium,[28] with or without NH_4^+ (5.6 mM), neat cDCE (0.3 mM) as the electron acceptor, hydrogen (207 μmol) as the electron donor, and acetate (5 mM) as the carbon source. All serum bottles were closed with butyl rubber stoppers (Geo-Microbial Technologies, Inc., Ochelata, OK) and incubated statically in the dark at 21 °C. The calculation of reductive dechlorination rates assumed that one chloride ion (Cl^-) is released per reductive dechlorination step and is reported as total μmol of Cl^- released per liter per day (i.e., μM Cl^- d^{-1}).

DNA Extraction. Biomass from PW4 and TC cultures was collected from 9 mL of culture suspension via vacuum filtration onto Durapore membranes (47 mm diameter, 0.22 μm pore size, Millipore, Billerica, MA). Biomass from 100 mL of well PW4 groundwater was collected with 0.22 μm Millipore Sterivex-GP cartridges, as described.[29] DNA was extracted from collected biomass or 0.5 g of TC sediment using the MoBio power soil DNA isolation kit (MO BIO, Carlsbad, CA), according to the manufacturer's recommendations. DNA was quantified using the Qubit dsDNA BR Assay (Life Technologies, Carlsbad, CA) and stored at −80 °C.

RNA Extraction, Purification, and Reverse Transcription. For RNA extraction, biomass from 15 mL of TC or PW4 cultures was collected via vacuum filtration onto 0.22 μm Durapore membrane filters and placed into 2 mL vials with 0.25 mL RNAlater (Qiagen, Germantown, MD). Total RNA was extracted from frozen filters using the RNeasy Mini Kit (Qiagen, Valencia, CA), quantified using the Qubit RNA BR Assay (Life Technologies), and processed as described.[30] DNA was removed using the Qiagen RNase-free DNase Set before

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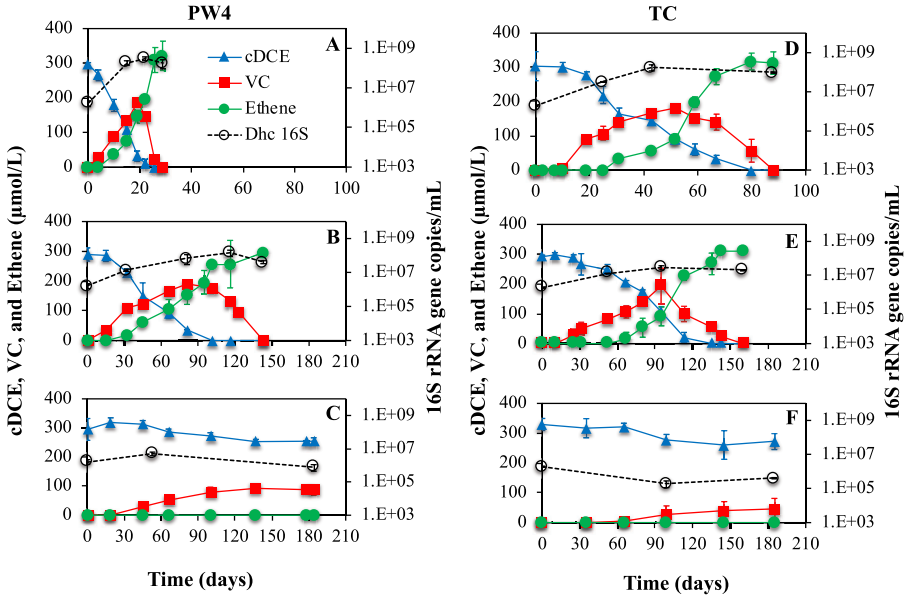

Figure 1. cDCE dechlorination and *Dhc* cell growth in PW4 and TC culture incubations. (A) PW4 cultures with NH_4^+, (B) PW4 cultures without NH_4^+, and (C) PW4 cultures with argon headspace (without NH_4^+ and N_2). (D) TC cultures with NH_4^+, (E) TC cultures without NH_4^+, and (F) TC cultures with argon headspace (without NH_4^+ and N_2). The data represent the average of triplicate measurements each obtained from three independent experiments. Error bars represent standard deviations (not shown if they are smaller than the symbols).

cDNA was obtained with the Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA).[30] To estimate RNA loss, 2 μL (2×10^{10} copies) of luciferase control mRNA (Promega, Madison, WI) was added as an internal standard after the cell lysis step.[30] The mRNA recovery ranged from 5 to 35% between samples.

Quantitative Polymerase Chain Reaction (qPCR). Established TaqMan qPCR assays were used to enumerate total bacterial 16S rRNA, *Dhc* 16S rRNA, *tceA*, *bvcA*, *vcrA* genes, and transcripts[31] as well as luciferase transcripts.[32] New primer/probe combinations were designed to amplify Cornell-type *Dhc* 16S rRNA and *Dhc* nitrogenase genes and transcripts (Table 1). qPCR calibration curves used triplicate 10-fold serial dilutions of the plasmid DNA carrying a single copy of the gene of interest (see the Supporting Information (SI) for details). The known *Dhc* strains harbor a single 16S rRNA gene and single-copy *nif* operons, and the gene copy numbers and the *Dhc* cell numbers are reported interchangeably. Transcript copy numbers are reported on a per gene basis as transcript-to-gene ratio (TGR) values.

16S rRNA Gene Amplicon Sequencing and Analysis. 16S rRNA gene fragments were amplified from purified DNA with the primer set 515F/806R targeting the V4 region of both bacterial and archaeal 16S rRNA genes.[33] A DNA library was prepared according to established procedures and sequenced on an Illumina MiSeq platform.[33] Sequencing data were analyzed using the QIIME v.1.9.1 software package[34] (see the SI for details).

Analytical Procedures. Chlorinated ethenes, ethene, and methane were analyzed by injecting 100 μL of headspace samples into an Agilent 7890A gas chromatograph equipped with a flame ionization detector and a DB-624 capillary column (60 m length x 0.32 mm i.d., 18 μm film thickness). Standard curves were prepared as described.[6]

Proteomics Analysis and Protein Identification in PW4 Groundwater and Enrichment Cultures. Biomass from 50 mL of PW4 enrichment cultures ($\pm NH_4^+$) and from 1000 mL of well PW4 groundwater was collected on duplicate 0.22 μm sterile Sterivex-GP cartridges, and 2 mL of RNAlater was added as a preservative. Sterivex cartridges of field samples were shipped on ice via express carrier and immediately stored at −80 °C. Total proteins were extracted from the biomass using a detergent-based extraction protocol and subjected to nanoLC−MS/MS analysis following established procedures (see the SI for details).[35,36] For protein identification, the raw spectra were searched against an assembled database of genomes representing aquifer microbial communities.[36] For data analysis, spectral counts of identified peptides were normalized to obtain the normalized spectral abundance factor (NSAF) and used to compare protein expression across samples and time points.[37]

RESULTS

Effects of NH_4^+ Availability and *Dhc* Dechlorination Activity and Growth. PW4 cultures dechlorinated cDCE to ethene regardless of NH_4^+ amendment. However, a 5-fold increase in dechlorination rates (i.e., 3.82 ± 0.48 vs 20.62 ± 1.58 μM Cl^- d^{-1}) was observed when NH_4^+ was added (Figure 1A,B). Accordingly, the time required for complete cDCE to ethene dechlorination was substantially longer in incubations without NH_4^+ (i.e., 29.0 ± 2.4 vs 143 ± 3.5 days). *Dhc* 16S rRNA gene copy numbers (i.e., *Dhc* cell numbers) increased 200-fold from $(1.7 \pm 0.5) \times 10^6$ (cells introduced with the inoculum) to $(1.8 \pm 0.9) \times 10^8$ mL^{-1} after 29 days of incubations with NH_4^+, whereas the *Dhc* cell numbers increased less than 25-fold to $(4.1 \pm 0.8) \times 10^7$ mL^{-1} after 143 days without NH_4^+ amendment. Complete cDCE to ethene dechlorination also occurred in TC cultures regardless of NH_4^+ amendment (Figure 1D,E), but without NH_4^+, a 2-fold reduction in dechlorination rates extended the time required to achieve complete dechlorination (i.e., 88 ± 5 vs 160 ± 7 days). In incubations with NH_4^+, the abundance of *Dhc* 16S rRNA genes increased from $(1.93 \pm 0.15) \times 10^6$ (cells introduced with the inoculum) to $(9.6 \pm 0.8) \times 10^7$ over an

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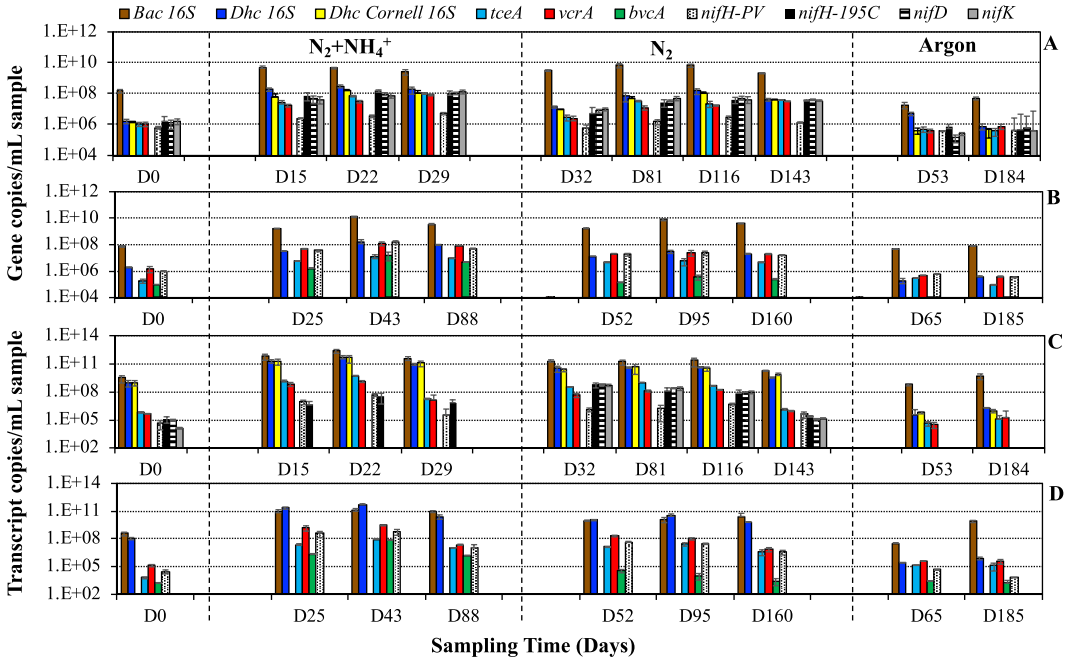

Figure 2. Changes in gene and transcript abundances in PW4 (A and C) and TC (B and D) cultures with and without NH_4^+ over the course of cDCE dechlorination. 1st column: day 0, 2nd column: incubations with NH_4^+, 3rd column: incubations without NH_4^+, 4th column: incubations with argon headspace (without NH_4^+ and N_2). The data represent the average of triplicate measurements each obtained from three independent experiments. Error bars represent standard deviations (not shown if they are smaller than the symbols).

88-day incubation period required to achieve complete cDCE dechlorination to ethene. In incubations without NH_4^+, *Dhc* cell numbers of $(2.0 \pm 0.2) \times 10^7$ mL^{-1} were measured after 160 days when dechlorination to ethene was complete. In PW4 and TC incubation vessels without NH_4^+ and argon headspace (no N_2 present), no more than 17% of the initial amount of cDCE was reduced to VC and no ethene was produced. The observed dechlorination was attributed to carry over of fixed N or possible intrusion of N_2 gas through the stopper. Slight increases in *Dhc* cell numbers were observed, but titers declined at the end of the incubation period (Figure 1C–F). Complete cDCE-to-ethene dechlorination occurred in pure cultures of *Dhc* strain BAV1 or strain GT in approximately 40 days in the medium supplemented with NH_4^+ (data not shown). In contrast, no growth or reductive dechlorination occurred without NH_4^+ consistent with the absence of *nifD* and *nifK* (i.e., incomplete *nif* operons) in *Dhc* strains affiliated with the Pinellas group.[25]

Effect of NH_4^+ Availability on Biomarker Gene Abundances. The *Dhc* cells detected in groundwater from well PW4 belonged to the Cornell group (Figure 2A, 1st column), and *Dhc* of the Pinellas/Victoria groups were either present below the qPCR detection limit of 10^2 16S rRNA gene copies L^{-1} or absent. In PW4 cultures grown with 0.3 mM cDCE without NH_4^+, the final number of total *Dhc* cells of $(4.1 \pm 0.8) \times 10^7$ equaled the number of Cornell-type *Dhc* (Figure 2A, 3rd column). In the presence of NH_4^+, PW4 cultures produced $(1.8 \pm 0.9) \times 10^8$ *Dhc* cells following cDCE dechlorination to ethene but only about 50% ($(9.8 \pm 5.3) \times 10^7$ copies mL^{-1}) were Cornell-type *Dhc*. Apparently, non-Cornell-type *Dhc* strains, presumably lacking the ability to fix N_2, responded to the supplementation with NH_4^+. In the TC cultures, *Dhc* Cornell 16S rRNA genes were not detected (Figure 2B).

The reductive dehalogenase (RDase) genes *tceA* and *vcrA* were detected in PW4 cultures regardless of NH_4^+ amendment. In PW4 incubations with NH_4^+, *tceA* and *vcrA* gene copy numbers had increased about 95- and 77-fold, respectively (Figure 2A, 2nd column), when cDCE had been completely dechlorinated to ethene, while without NH_4^+, lower increases of 37-fold (*tceA*) and 28-fold (*vcrA*) were observed (Figure 2A, 3rd column). The total abundances of *vcrA* genes $((8.3 \pm 0.1) \times 10^7$ and $(3.1 \pm 0.1) \times 10^7$ mL^{-1} with and without NH_4^+, respectively) were within 76–78% of the total Cornell-type *Dhc* cell numbers ($(1.1 \pm 0.5) \times 10^8$ and $(4.1 \pm 0.3) \times 10^7$ mL^{-1} with and without NH_4^+, respectively) indicating that at least one Cornell strain harbors *vcrA*. In TC cultures with NH_4^+, *vcrA* and *tceA* gene copies increased 60- and 50-fold, respectively (Figure 2B, 2nd column), whereas substantially lower increases were observed in cultures without NH_4^+ (Figure 2B, 3rd column). The *bvcA* RDase gene was only present in TC cultures, and a 50-fold increase to $(4.86 \pm 0.11) \times 10^6$ *bvcA* genes mL^{-1} was observed in incubations with NH_4^+ (Figure 2B, 2nd column). In contrast, the increase was only 3-fold in incubations without NH_4^+ (Figure 2B, 3rd column), indicating that NH_4^+ limitation impacted *Dhc* strains carrying *bvcA* more than other *Dhc* strains.

The Cornell-type *nif* genes *nifD*, *nifK*, and *nifH* (*nifH-195C*) were solely detected in PW4 cultures, whereas the Pinellas/Victoria-type *Dhc nifH* gene (*nifH-PV*) was present both in PW4 and TC cultures regardless of NH_4^+ amendments (Figure 2A,B). When PW4 cultures were incubated with NH_4^+, the copy number of Cornell-type *nifD*, *nifK*, and *nifH-195C*, and Pinellas/Victoria-type *nifH-PV* genes increased by 87-, 81-, 70-, and 8-fold, respectively, with copies ranging between $(5.19 \pm 0.87) \times 10^6$ and $(1.34 \pm 0.36) \times 10^8$ mL^{-1}. In contrast, in incubations without NH_4^+, the overall increase of those genes was about 34-, 22-, 22-, and 2-fold, respectively, with copies ranging between $(1.37 \pm 0.13) \times 10^6$ and $(4.09 \pm$

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Table 2. Microbial Community Composition and Relative Abundance (%) based on 16S rRNA Gene Amplicon Sequencing[a]

Phyla	Taxon[b]	PW4 Culture			TC Culture		
		GW	With NH_4^+ Day 29	Without NH_4^+ Day 143	Sediment	With NH_4^+ Day 88	Without NH_4^+ Day 160
Crenarchaeota[c]	f_*Cenarchaeaceae*	<0.1	-	-	2.1	-	-
Euryarchaeota[c]	*Methanocorpusculum*	7.6	1.3	0.2	<0.1	<0.1	<0.1
	Candidatus Methanoregula	<0.1	-	-	7.1	<0.1	<0.1
	Methanospirillum	<0.1	-	-	<0.1	1.6	0.8
	Methanosaeta	<0.1	-	-	3.5	<0.1	<0.1
Actinobacteria	f_*Coriobacteriaceae*	40.7	<0.1	<0.1	<0.1	<0.1	<0.1
Bacteroidetes	o_*Bacteroidales*	7.6	28.0	6.1	20.6	40.7	32.4
	f_*Cytophagaceae*	<0.1	<0.1	-	2.8	<0.1	<0.1
	o_*Saprospirales*	<0.1	-	-	2.6	<0.1	<0.1
Chlorobi	f_*Ignavibacteriaceae*	-	-	-	4.3	<0.1	<0.1
Chloroflexi	*Dehalococcoides*	1.0	4.6	1.5	<0.1	6.7	2.3
Deferribacteres	f_*Deferribacteraceae*	2.8	<0.1	-	-	<0.1	<0.1
Firmicutes	*Bacillus*	1.8	<0.1	<0.1	<0.1	<0.1	-
	Clostridium	1.8	36.9	<0.1	<0.1	1.1	0.1
	Proteiniclasticum	<0.1	<0.1	<0.1	<0.1	14.7	33.6
	f_*Clostridiaceae*	<0.1	-	-	<0.1	6.2	0.6
	f_*EtOH8*	-	-	<0.1	<0.1	1.9	0.1
	Acetobacterium	0.2	1.9	41.7	0.1	1.4	2.6
	Dehalobacter_ Syntrophobotulus	1.0	3.7	0.9	0.1	0.1	0.1
	Syntrophomonas	-	<0.1	<0.1	<0.1	2.5	0.1
	Anaeromusa	<0.1	<0.1	<0.1	<0.1	0.4	6.5
	Acidaminobacter	<0.1	<0.1	-	<0.1	4.9	2.3
	Fusibacter	0.6	1.8	<0.1	<0.1	0.5	0.3
	PSB-M-3	<0.1	<0.1	-	0.1	2.2	5.9
Nitrospirae	o_*Nitrospirales*	<0.1	<0.1	<0.1	5.4	<0.1	<0.1
Proteobacteria	f_*Rhodobacteraceae*	12.0	5.2	0.1	<0.1	<0.1	<0.1
	f_*Comamonadaceae*	<0.1	<0.1	<0.1	15.1	<0.1	<0.1
	Desulfomicrobium	<0.1	1.6	0.1	<0.1	<0.1	<0.1
	Desulfovibrio	4.5	<0.1	<0.1	<0.1	<0.1	<0.1
	Geobacter	<0.1	<0.1	<0.1	4.6	1.4	0.5
	Anaeromyxobacter	<0.1	-	-	2.3	-	<0.1
	Syntrophus	<0.1	2.2	0.1	5.0	3.5	2.8
	Sulfurospirillum	3.5	7.0	45.7	<0.1	0.2	0.6
	o_*Alteromonadales*	1.2	<0.1	<0.1	4.7	<0.1	-
	Crenothrix	<0.1	<0.1	-	2.9	<0.1	<0.1
Spirochaetes	o_*PL-11B10*	<0.1	-	-	<0.1	0.4	1.5
	f_*Sphaerochaetaceae*	<0.1	0.2	1.8	0.3	0.1	1.6
Synergistetes	*vadinCA02*	1.3	1.9	0.7	<0.1	0.2	0.2
Tenericutes	*o_RF39*	4.0	-	-	<0.1	<0.1	-
Verrucomicrobia	f_*auto67_4W*	<0.1	-	-	3.2	<0.1	<0.1
Unassigned	Unassigned[d]	2.7	-	-	<0.1	<0.1	<0.1

Relative sequence abundance (%): 0.1 45.7

[a]Shown are the most abundant phyla and taxa detected in well PW4 groundwater used to establish PW4 enrichment cultures in TC sediment used to establish TC enrichment cultures and in PW4 and TC enrichment cultures with or without NH_4^+. [b]OTUs not assigned to any genus classified either at family (f) or order (o) level. [c]Archaea. [d]Sequences that could not be assigned to any of these taxonomic ranks. "-": Not detected.

0.44) × 10^7 mL^{-1}. The copy numbers of *nifH-PV* genes in PW4 cultures were 10−15-fold lower than that of Cornell-type *nif* genes indicating the dominance of Cornell-type *Dhc* cells over Pinellas/Victoria-type *Dhc* cells, which possibly reflects the relative abundance of these *Dhc* types in fixed N-limited PW4 groundwater.

The detection of *vcrA*, *tceA*, and *bvcA* in both PW4 and TC cultures suggests the involvement of multiple *Dhc* strains in reductive dechlorination, because these genes have not been found on a single *Dhc* genome. In contrast, the *tceA* gene has been found on Cornell group genomes (i.e., strain ANAS1 and strain 195) and on Pinellas group genomes (i.e., strain KB1/VC and strain FL2). Only the *vcrA* RDase gene has been detected in genomes of *Dhc* strains belonging to all three groups (i.e., Cornell, Pinellas, and Victoria), whereas *bvcA* appears restricted to Pinellas group genomes.[38,39]

Consistent with the absence of Cornell-type *Dhc* 16S rRNA genes in TC cultures (Figure 2B), Cornell-type *Dhc nifD* and *nifK* genes were not detected. In TC cultures, the numbers of *nifH-PV* genes increased from (1.01 ± 0.18) × 10^6 to (5.03 ± 0.44) × 10^7 and to (1.64 ± 0.44) × 10^7 copies mL^{-1} in the presence and in the absence of NH_4^+, respectively. Overall, similar increasing trends were observed in the total *Dhc* 16S rRNA gene and the RDase gene abundances in PW4 and TC

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Environ. Sci. Technol. 2019, 53, 14548−14558

Table 3. Relative Peptide Abundance of Selected *Dhc*-Type Proteins Analyzed through Global Proteomics in PW4 Groundwater (Used to Establish PW4 Enrichment Cultures) and in PW4 Culture Incubations with or without NH_4^+ during cDCE-to-Ethene Dechlorination[a]

Description	GW	With NH_4^+			Without NH_4^+			
		D15	D22	D29	D32	D82	D116	D143
Nitrogen regulatory protein P-II[b]	0	0	0	0	1320	602	102	380
Nitrogen regulatory protein P-II[b]	0	0	0	0	478	444	74	257
Nitrogen regulatory protein P-II[b]	0	0	0	0	112	16	2	12
Nitrogenase reductase (nitrogenase iron protein), NifH[b]	3	0	0	0	357	115	20	43
Nitrogenase molybdenum−iron protein α chain, NifD[b]	0	0	0	0	68	26	5	17
Nitrogenase molybdenum−iron protein β chain, NifK[b]					21	12	3	0
Ammonium transporter, AmtB[b]	0	0	0	0	24	28	7	142
Molybdenum ABC transporter, periplasmic molybdate-binding protein[b]	0	0	0	0	54	61	10	40
Glutamine synthetase, type I[b]	228	81	56	39	320	435	355	424
Glutamine synthetase, type I[c]	0	0	0	0	103	222	245	136
Glutamine synthetase, glna gene product[c]	0	49	95	141	4	0	0	0
Glutamate synthase-like protein protein, α subunit-like protein[d]	161	7	15	11	19	16	1	9
Glutamate synthase, α subunit[c]	2	5	11	18	39	35	5	40
Glutamate synthase-like protein, gltb-like fragment[c]	0	0	12	24	54	42	30	53
Glutamate synthase (NADPH), homotetrameric[c]	0	0	0	11	0	3	0	4
Glutamate synthase (NADPH), homotetrameric[d]	37	23	10	0	11	0	1	7
Trichloroethene reductive dehalogenase[b]	180	144	148	171	63	165	26	37
Vinyl chloride reductive dehalogenase[c]	334	280	346	436	285	386	62	156
Reductive dehalogenase[e]	335	280	342	431	269	386	63	220
Reductive dehalogenase[b]	96	12	31	37	21	31	4	34
Reductive dehalogenase[b]	2	0	0	0	0	24	5	18
Reductive dehalogenase homologous protein RdhA8[f]	0	0	0	0	0	4	0	0
Reductive dehalogenase homologous protein RdhA9[f]	0	0	0	0	0	3	0	0
Reductive dehalogenase[c]	0	5	0	0	13	0	2	0
Reductive dehalogenase[c]	0	0	0	0	0	3	0	0
MraZ[b]	0	0	0	0	0	16	7	41
MraZ[d]	0	0	0	0	0	0	0	9
Methylglyoxal synthase (MGS)[b]	0	0	0	0	0	20	7	0

[a]Spectral counts of identified peptides were normalized to obtain the normalized spectral abundance factor (NSAF). For better visualization, the NSAF values were multiplied by a constant number (100 000). Peptide of the protein matches related protein of: [b]*Dhc* strain 195; [c]all *Dhc* strains; [d]*Dhc* strain VS; [e]*Dhc* strain GT; and [f]*Dhc* strain CBDB1. Abbreviations: D: Day, *Dhc*: *Dehalococcoides mccartyi*.

cultures regardless of NH_4^+ amendment during cDCE-to-ethene dechlorination.

Effects of NH_4^+ Availability on Biomarker Gene Expression. In PW4 cultures, the *tceA* gene was the most highly transcribed RDase. The expression level increased to $(5.26 \pm 0.4) \times 10^9$ transcripts mL^{-1} at day 22 with NH_4^+ amendment, before declining to $(1.74 \pm 0.27) \times 10^7$ transcripts mL^{-1} at day 29, when all chlorinated ethenes had been dechlorinated to ethene (Figure 2C, 2nd column). Transcript-to-gene ratios (TGRs) for *tceA* were 0.6 at day 0 (0% cDCE consumed), 50 at day 15 (about 75% of cDCE consumed), 75 at day 22 (about 90% of cDCE consumed), and 0.2 at day 29 (all chlorinated ethenes dechlorinated to ethene) (Figure S2A). A similar trend was observed in PW4 cultures without NH_4^+ with TGRs for *tceA* ranging between 0.03 and 114 over the 29-day incubation period (with lower TGRs observed when complete dechlorination had occurred and higher TGRs observed during cDCE dechlorination). *vcrA* transcripts followed a similar decreasing trend as dechlorination progressed with TGRs ranging between 0.4 and 41 in cultures with NH_4^+ and between 0.02 and 17 in cultures without NH_4^+ (Figure S2A). In TC cultures, the *vcrA* gene was the most highly transcribed RDase (Figure 2D, 3rd column) with TGRs ranging between 0.3 and 34 (with NH_4^+) and 0.4 and 10 (without NH_4^+). TGRs were lower when the chlorinated electron acceptors had been consumed (i.e., dechlorination to ethene was complete), and higher TGRs were observed during active dechlorination (Figure S2B). Similar trends were observed for the expression patterns of the *tceA* and *bvcA* RDase genes.

The Cornell-type *nifD* and *nifK* transcripts were only detected in PW4 cultures without NH_4^+ (Figure 2C, 3rd column), highlighting the role of these genes in N_2 fixation. As dechlorination proceeded, similar to the RDase genes, the Cornell-type *nif* genes in PW4 cultures without NH_4^+ followed a decreasing trend with TGRs ranging between 51 and 108 with 20% of cDCE consumed, declining to 4−7 with 90% of cDCE consumed, and declining further to 0.003−0.006 when complete dechlorination to ethene had been achieved (Figure S2A). In TC cultures, transcripts of the *nifH-PV* gene also declined with TGRs ranging between 0.2 and 11 (with NH_4^+) and 0.2 and 3 (without NH_4^+) with the lowest ratio observed at the completion of cDCE-to-ethene dechlorination. Similar to *nifH-195C* gene expression patterns in PW4 cultures, *nifH-PV* transcripts were measured in TC cultures regardless of NH_4^+ amendment, suggesting that the expression of these genes was not regulated by NH_4^+. Taken together, all monitored *Dhc* genes were expressed at higher levels in the presence of NH_4^+. About 1 to 2-orders of magnitude differences in transcriptional levels were observed with an

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Environ. Sci. Technol. 2019, 53, 14548−14558

initially high increase followed by a decline during the course of electron acceptor (i.e., cDCE and VC) consumption in both PW4 and TC cultures (Figure 2C,D).

Effects of NH_4^+ on the Microbial Community. The 16S rRNA gene amplicon sequencing data revealed community differences associated with PW4 groundwater collected within a contaminated aquifer in Australia and TC river sediment collected in Knoxville, TN where infiltrating groundwater is contaminated with chlorinated solvents.[27] The analysis of PW4 and TC enrichment cultures indicated that NH_4^+ amendment impacted the microbial community composition during cDCE dechlorination. In PW4 well groundwater, *Actinobacteria* (42%), *Proteobacteria* (22%), *Firmicutes* (9%), and *Bacteroidetes* (8%) were the most abundant bacterial phyla (Table 2), whereas the phylum *Chloroflexi* (1%) was less abundant. Over the course of repeated transfers of the PW4 enrichment culture to the fresh medium with cDCE provided as the electron acceptor, *Actinobacteria* diminished, while the relative abundances of *Firmicutes* and *Chloroflexi* increased regardless of NH_4^+ amendment. At the genus level, in PW4 culture incubations without NH_4^+, *Sulfurospirillum* and *Acetobacterium* substantially increased in abundance when compared to their levels in PW4 groundwater (i.e., increases from 4 to 46% and from 1 to 42%, respectively), suggesting that members of these genera contributed to N_2 fixation. Sequences affiliated with *Clostridium* (37%) and unclassified *Bacteroidales* (28%) were most abundant in cultures amended with NH_4^+ (day 29), whereas their abundances decreased without NH_4^+. Consistent with the qPCR results (Figure 2A), the abundance of *Dhc* increased regardless of NH_4^+ amendment (Table 2), albeit higher *Dhc* cell numbers were attained with NH_4^+ (i.e., $(1.8 \pm 0.9) \times 10^8$ vs $(4.1 \pm 0.8) \times 10^7$ copies mL^{-1}).

The TC sediment community was dominated by *Proteobacteria* (44%) and *Bacteroidetes* (26%), while *Chloroflexi* and *Firmicutes* were less abundant (<1%) (Table 2). Over the course of repeated transfers of the TC enrichment culture to the fresh medium with cDCE provided as the electron acceptor, the relative abundance of *Proteobacteria* declined, whereas *Bacteroidetes* and *Firmicutes* (i.e., *Proteiniclasticum*) increased substantially regardless of NH_4^+ amendment (Table 2). Similar to PW4 culture incubations, the abundance of *Dhc* increased regardless of NH_4^+ amendment (Table 2) albeit the increase was more pronounced in cultures with NH_4^+ (6.7 vs 2.3% of sequences), which was consistent with the qPCR results (Figure 2B).

Detection of Biomarker Proteins in the PW4 Enrichment Cultures and Source Groundwater. TceA (matching *Dhc* strain 195) and VcrA (matching *Dhc* strain VS) were detected in relative abundances corresponding to their transcription levels (Figure 2C,D). The *tceA* gene was transcribed 1.2- to 8-fold higher than the *vcrA* gene, whereas the relative spectral counts of VcrA peptides (NSAF of 280−436 and 62−386 with or without NH_4^+, respectively) were 2- to 4-fold higher compared to the counts of the TceA peptides (Table 3). In addition to TceA and VcrA, peptides of seven other *Dhc*-type RDase proteins were detected in PW4 samples, albeit at lower abundances, while peptides of non-*Dhc* RDases were not detected in PW4 enrichment cultures (Table S2A,B).

The proteomic analysis detected peptides of the nitrogenase proteins NifD, NifK, and NifH and three homologs of the nitrogen regulatory protein P-II matching those of *Dhc* strain 195 only in PW4 cultures without NH_4^+ (Table 3). Relative abundances of nitrogenase peptides were higher at the beginning of cDCE dechlorination (day 32) compared to those near completion (day 143) (Table 3) and, thus, corresponded to their respective transcript levels (Figure 2C,D). Similarly, peptides of the NH_4^+ transporter protein AmtB were solely detected in PW4 cultures without NH_4^+. Peptides matching *Dhc*-type glutamine and glutamate synthase were expressed in PW4 cultures regardless of NH_4^+ amendment (Table 3). Despite the detection of *nifH* transcripts in cultures with NH_4^+, peptides of other nitrogenase proteins were not detected, indicating that *nifH* transcripts do not serve as biomarkers for N_2 fixation and that NH_4^+ regulated the expression of these proteins. Peptides of methylglyoxal synthase (MGS) and the transcriptional regulator MraZ matching those of *Dhc* strain 195 were detected only in the incubations without NH_4^+ (Table 3). MGS synthesizes methylglyoxal, a toxic electrophile, that can inhibit growth,[40] while MraZ synthesizes a conserved transcription factor implicated in the regulation of cell division,[41] suggesting these proteins have regulatory functions in Cornell-type *Dhc* strains. The proteomics analysis also detected peptides of non-*Dhc* nitrogenases in cultures without NH_4^+ but not in cultures with NH_4^+ (Table S2A). Other, non-*Dhc* and *Dhc* proteins that were differentially expressed during growth with lactate as the electron donor and cDCE as the electron acceptor are listed in Table S2A.

Effects of NH_4^+ Amendment on *Dhc* Activity and Biomarker Proteins in a Contaminated Groundwater Aquifer. Monitoring of well PW4 groundwater demonstrated stalled dechlorination with VC as the dominant chlorinated ethene (Figure S3) and a decline of *Dhc* 16S rRNA, *vcrA*, and *tceA* genes over time.[10] Proteomic analysis of groundwater samples revealed the expression of the *Dhc* nitrogenase proteins NifH and NifD (Table S3, for a complete list of proteins detected, see Table S2B), suggesting that *Dhc* were experiencing fixed N limitation. Total Kjeldahl N (TKN) and NH_4^+-N measurements (Table S4) supported this conclusion. Therefore, soluble Aquasol fertilizer containing urea was added to wells up-gradient of PW4. In response, elevated TKN and NH_4^+-N values (Table S4), enhanced dechlorination activity (Figure S3), and higher abundances of *Dhc* 16S rRNA, *vcrA*, and *tceA* genes were observed. Consistent with the qPCR results, the proteomic analysis demonstrated an increase of TceA and VcrA peptides (NSAF values of 0.8−0.9 before versus 2−3.8 after Aquasol fertilizer addition) as well as of non-*Dhc* RDase peptides such as tetrachloroethene reductive dehalogenase, PceA, of *Dehalobacter* sp. UNSWDHB and PceA of *Dehalobacter restrictus* DSM 9455 but a decrease of *Dhc* nitrogenase proteins NifH and NifD (NSAF values of 30.1 and 2.6 versus 10.3 and 0, respectively) after fertilizer addition (Table S3).

DISCUSSION

Effects of NH_4^+ on *Dhc* Growth and Reductive Dechlorination Performance. Groundwater aquifers are often oligotrophic (i.e., nutrient poor),[1−3] and fixed N limitation can negatively impact reductive dechlorination activity. NH_4^+ limitation impacted *Dhc* growth and reductive dechlorination activity and resulted in lower reductive dechlorination rates, longer time frames to complete dechlorination, and lower *Dhc* biomarker gene abundances in PW4 and TC enrichment cultures. The availability of NH_4^+ also affected the relative abundance of *Dhc* strains belonging to the Cornell versus the Pinellas/Victoria groups in PW4

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cultures, consistent with gene content and physiology attributed to Cornell versus Pinellas/Victoria group *Dhc*.[14,22−24,42,43] Cornell-type *Dhc* have an advantage over Pinellas/Victoria-type *Dhc* under NH_4^+-limiting conditions due to their N_2-fixing ability. N_2 fixation is an energetically demanding process, diverts electron flow and reduces energy gain, and, thus, impacts dechlorination activity, as was observed experimentally in PW4 cultures dominated by Cornell-type *Dhc*. Pinellas/Victoria-type *Dhc* lack the ability to fix N_2 but TC culture incubations without NH_4^+ also achieved complete cDCE-to-ethene dechlorination (Figure 2B), presumably due to the activity of diazotrophs present in the culture. This is a relevant observation indicating that non-N_2 fixing *Dhc* strains depend on community diazotrophs for supplying fixed N under N-limiting conditions.

Although the addition of fixed N (e.g., NH_4^+, Aquasol fertilizer) improved *Dhc* activity and reductive dechlorination performance, elevated NH_4^+-N concentrations (i.e., ≥0.5 g NH_4^+ L^{-1}) can decrease dechlorination rates, particularly the conversion of the crucial VC-to-ethene dechlorination step.[44] Furthermore, fixed N turnover in groundwater aquifers results in the formation of nitrous oxide (N_2O),[45,46] which is a potent inhibitor of reductive dechlorination activity and a possible cause for *c*DCE or VC stalls.[47] Therefore, the decision of fixed N addition requires careful evaluation and should be based on the presence of *Dhc* types (i.e., N_2-fixing Cornell-type *Dhc* versus non-N_2-fixing Pinellas/Victoria-type *Dhc*), the time frames to reach desirable remediation endpoints, and the ability to implement a rigorous monitoring regime to alert about potential dechlorination stalls due to elevated N_2O concentrations.[47]

Effects of NH_4^+ on Microbial Community Composition. Without fixed N additions to PW4 and TC cultures, bacterial groups characterized as diazotrophs increased in abundance. For example, species of *Sulfurospirillum*, *Acetobacterium*, and members of the *Spirochaetes* possess complete *nif* operons,[48] suggesting N_2 fixation capabilities. Furthermore, *Sulfurospirillum*, *Acetobacterium*, *Clostridium*, and *Spirochetes* are often found in groundwater contaminated with chlorinated ethenes[5,8,9] and have been implicated in supplying *Dhc* with essential nutrients such as hydrogen,[49,50] acetate,[50] and corrinoid.[51,52] *Proteiniclasticum*, a major population in the TC culture without NH_4^+, reportedly digests proteins[53] and releases nitrogenous metabolites (e.g., from necromass) that can fulfill the nutritional N requirement of *Dhc*. Since diazotrophs are common members of groundwater aquifer microbiomes, N_2 fixation will occur under fixed N-limiting conditions and nitrogenous metabolites will be generated and become available to *Dhc*. While this "do-nothing" approach may not result in the highest possible dechlorination rates, it saves the operational costs for fertilizer biostimulation and avoids the likelihood of stalled reductive dechlorination due to elevated N_2O concentrations as explained above. None of the 16S rRNA gene amplicon sequences derived from the enrichment cultures affiliated with the genus *Dehalogenimonas*. Members of this genus have been implicated in reductive dechlorination of chlorinated ethenes, and *Dehalogenimonas* sp. WBC-2 dechlorinates *trans*-1,2-dichloroethene to VC and '*Candidatus* Dehalogenimonas etheniformans' strain GP dechlorinates trichloroethene (TCE) to ethene.[54,55] A 16S rRNA-based survey demonstrated the broad distribution of *Dehalogenimonas* spp. in groundwater aquifers impacted with chlorinated ethenes, suggesting that members of this group contribute to reductive dechlorination and detoxification.[56] Based on the gene content of the five *Dehalogenimonas* genomes spp. (NCBI:txids 1217799, 943347, 1536648, 1839801, 552811), only *Dehalogenimonas* sp. strain WBC-2 (NCBI:txid 943347) has a complete *nif* operon and can potentially fix N_2.

Effects of NH_4^+ on Biomarker Gene and Transcript Abundances. *Dhc* share highly similar 16S rRNA gene sequences, but not all *Dhc* strains have the ability to dechlorinate cDCE and VC to ethene.[57] Accordingly, specific metabolic functions cannot be inferred from *Dhc* 16S rRNA gene-targeted approaches, and the presence of *Dhc* does not guarantee that VC is efficiently dechlorinated to ethene. RDase genes (i.e., *tceA, bvcA, and vcrA*) that encode RDase proteins with assigned functions can diagnose and demonstrate *Dhc* growth and specific dechlorination activities. Hence, efforts to monitor the responses of *Dhc* populations to treatment and evaluate bioremediation performance should include 16S rRNA gene and RDase gene analyses.[31] In PW4 cultures, *tceA* was consistently more abundant than *vcrA* regardless of NH_4^+ amendment, while the *bvcA* gene was not detected. In contrast, in TC cultures, the abundance of the *vcrA* gene was highest followed by *tceA* and *bvcA* gene abundances regardless of NH_4^+ amendment. Despite the presence of the *bvcA* gene in TC cultures, this gene was only upregulated in incubations with NH_4^+. The results indicate that the effect of NH_4^+ limitation was much more pronounced at the transcriptional level than at the gene level. *Dhc* strains harboring *bvcA*, a gene that encodes BvcA responsible for cDCE and VC reductive dechlorination, were most sensitive to NH_4^+ availability (Figure 1), suggesting that NH_4^+ limitation impacts *Dhc* strains lacking complete *nif* operons differently.

RDase gene transcripts declined as chlorinated electron acceptors (i.e., cDCE, VC) were depleted regardless of NH_4^+ amendments (Figure 2C,D). The TGRs were within the ranges reported in prior studies performed with cDCE-degrading *Dhc* enrichment cultures (i.e., TGRs between 10 and 100),[58−60] suggesting a direct link between TGRs and electron acceptor availability. A similar declining trend in TGR values was observed in the transcript numbers of *nif* genes as dechlorination progressed. Interestingly, *nifH-PV and nifH-195C* transcripts were detected in PW4 cultures with NH_4^+, but *nifK* and *nifD* transcripts were only detected in cultures without added NH_4^+, suggesting that *nifK* and *nifD*, but not *nifH* transcripts, are indicators of *Dhc* nitrogenase activity. These observations are consistent with the grouping of the Pinellas/Victoria *nifH* with cluster IV *nifH* sequences (i.e., homologs of diverse *nifH* gene sequences) not involved in N_2 fixation.[18,61]

Detection of Biomarker Proteins during NH_4^+ Limitation. Monitoring merely the presence/absence of organism- and process-specific biomarker genes is generally not sufficient to imply microbial activity,[57] and expression data can provide more direct evidence of activity. A factor complicating transcript analysis is the inherent instability and fast decay of RNA.[62] The analysis of proteins in groundwater has become feasible, and this measurement can provide additional evidence about the microbial process of interest.[63,64] Of note, DNA-based tools would not have uncovered that fixed N availability limited reductive dechlorination rates in the area surrounding well PW4, highlighting the value of untargeted proteomics analysis.

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The detection of several *Dhc* proteins implicated in N fixation, N metabolism, and the control of growth (i.e., MGS and MraZ) in incubations without NH_4^+ suggests their potential role as biomarker proteins for *Dhc* activity. Nitrogenase proteins were only identified in PW4 cultures without NH_4^+ and in groundwater samples of well PW4 prior to fertilizer additions, indicating that *Dhc* NifK and NifD can serve as activity biomarkers for N_2 fixation. The observation that NifH was expressed in *Dhc* cultures with NH_4^+ indicated that this protein is not a useful biomarker for N_2 fixation, which is consistent with the results of the transcript analysis.

The main bacterial pathway for NH_4^+ incorporation into amino acids, including in *Dhc*, is through glutamine synthetase (GS) encoded by *glnA* and glutamate:2-oxoglutarate aminotransferase (GOGAT) encoded by *gltA*, also known as the GS-GOGAT cycle.[15,42] The detection of the peptides of *Dhc*-type GS and glutamate synthase (Table 3) suggests that non-N_2 fixing *Dhc* strains acquired NH_4^+, possibly derived from necromass. All three homologs of the N regulatory protein P-II control the formation of GS and were detected in PW4 cultures without NH_4^+.[15,65] Two of three genes that encode the N regulatory protein P-II are located within the *nif* operon and their expression reportedly coupled to the *nif* genes.[42] The third P-II gene is co-located with GS-GOGAT pathway genes (*glnA* and *gltA*) and adjacent to an NH_4^+ transporter gene (*amtB*). The NH_4^+ transporter protein AmtB is required for growth under NH_4^+-limiting conditions[66] and was only detected in PW4 cultures without NH_4^+. These findings suggest that the N regulatory protein P-II and AmtB can also serve as potential biomarkers for NH_4^+ or fixed N limitations.

Implications for Enhanced in Situ Bioremediation. Enhanced anaerobic bioremediation through biostimulation with or without bioaugmentation has achieved extensive reductions in the concentrations of chlorinated ethenes at many chlorinated solvent-impacted sites.[12,67] To sustain in situ bioremediation, the nutritional requirements of *Dhc* (and *Dehalogenimonas* spp.) must be met and the buildup of inhibitory chemicals has to be avoided.[20] Biostimulation generally involves the addition of fermentable substrates with the goal of increasing the flux of hydrogen. The results from this study illustrate that NH_4^+ limitation impairs *Dhc* reductive dechlorination activity, and biostimulation with fixed N can overcome this bottleneck. However, possible negative feedback on *Dhc* reductive dechlorination activity must be considered, including inhibition by elevated NH_4^+ following fertilizer addition or by N_2O resulting from increased N turnover.[44,47] Without fixed N biostimulation, native diazotrophs will fix N_2 under N-limiting conditions and provide fixed N to organisms lacking this ability, including *Dhc* of the Pinellas and Victoria groups. While this "do nothing" approach will not result in the highest possible reductive dechlorination rates, it does not require fertilizer additions and prevents unintended negative consequences, including N_2O emissions to the atmosphere (i.e., N_2O is a strong greenhouse gas with ozone destruction potential) and inhibition of *Dhc* dechlorination activity leading to cDCE and VC stalls.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.9b04463.

Experimental approaches, including qPCR, 16S rRNA gene amplicon sequencing and analysis, and proteomic workflows; Overview of growth conditions (Table S1); relative peptide abundances of selected proteins analyzed through global proteomics in groundwater collected from well PW4 (Table S3); N measurements in groundwater collected from well PW4 between November, 2013 and September, 2014 (Table S4); phylogenetic tree of *Dhc* isolates divided into the three recognized *Dhc* groups (i.e., Pinellas, Victoria, and Cornell) (Figure S1); changes in TGRs over time in PW4 and TC culture incubations ($\pm NH_4^+$) (Figure S2); chlorinated ethenes and ethene measurements in well PW4 groundwater, and the abundance of the total *Dhc* 16S rRNA and RDase genes in groundwater samples collected from well PW4 (Figure S3) (PDF)

Complete list of peptides representing bacterial and archaeal proteins detected in samples of groundwater well PW4 (located within a chlorinated solvent plume and used to establish PW4 enrichment cultures) and of PW4 culture incubations ($\pm NH_4^+$) (Table S2A); complete list of peptides representing bacterial and archaeal proteins detected in samples of groundwater well PW4 collected between November, 2013 and October, 2014 (Table S2B) (xlsx)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: frank.loeffler@utk.edu. Phone: (865) 974-4933.

ORCID

Devrim Kaya: 0000-0002-7115-0845

Robert L. Hettich: 0000-0001-7708-786X

Frank E. Löffler: 0000-0002-9797-4279

Present Address

○Growcentia, 500 E Vine Dr, Fort Collins, Colorado 80524, United States (K.C.).

Notes

The authors declare no competing financial interest.

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