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A Slow-Release Substrate Stimulates Groundwater Microbial Communities for Long-Term in Situ Cr(VI) Reduction

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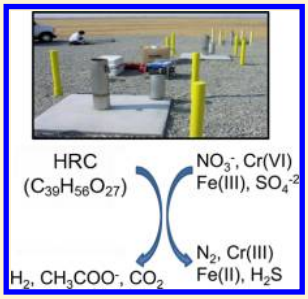
Supporting Information

ABSTRACT: Cr(VI) is a widespread environmental contaminant that is highly toxic and soluble. Previous work indicated that a one-time amendment of polylactate hydrogen-release compound (HRC) reduced groundwater Cr(VI) concentrations for >3.5 years at a contaminated aquifer; however, microbial communities responsible for Cr(VI) reduction are poorly understood. In this study, we hypothesized that HRC amendment would significantly change the composition and structure of groundwater microbial communities, and that the abundance of key functional genes involved in HRC degradation and electron acceptor reduction would increase long-term in response to this slowly degrading, complex substrate. To test these hypotheses, groundwater microbial communities were monitored after HRC amendment for >1 year using a comprehensive functional gene microarray. The results showed that the overall functional composition and structure of groundwater microbial communities underwent sequential shifts after HRC amendment. Particularly, the abundance of functional genes involved in acetate oxidation, denitrification, dissimilatory nitrate reduction, metal reduction, and sulfate reduction significantly increased. The overall community dynamics was significantly correlated with changes in groundwater concentrations of microbial biomass, acetate, NO_3^-, Cr(VI), Fe(II) and SO_4^{2-}. Our results suggest that HRC amendment primarily stimulated key functional processes associated with HRC degradation and reduction of multiple electron acceptors in the aquifer toward long-term Cr(VI) reduction.



INTRODUCTION

The widespread use of chromium (Cr) in industry and weapons production (e.g., corrosion inhibition, wood preservation, leather tanning) has caused soil and water contamination at many sites throughout the world.[1,2] Cr(VI), the most common valence state in oxidizing environments, is highly toxic (e.g., mutagenic, carcinogenic) and soluble, presenting a hazard to the environment.[3] Particularly, the spread of Cr(VI) through groundwater threatens aqueous ecosystems and drinking water resources. For instance, at the U.S. Department of Energy's (DOE) Hanford (WA) site, the release of 1.7 trillion liters of nuclear waste introduced substantial Cr(VI) into the groundwater.[4] After decades of natural attenuation and recent pump-and-treat applications, high concentrations of Cr(VI) (~100 μg/L) still persist at the 100-H Area along the groundwater flow path from the point of initial contamination to the Columbia River,[5] the largest river in the Pacific Northwest region of North America. Since groundwater must meet the U.S. Environmental Protection Agency's ambient Water Quality Standard (22 μg/L Cr(VI)) before discharging into the river (http://www.hanford.gov/page.cfm/soilgroundwater), there is an urgent need to develop strategies for long-term remediation of this contaminated groundwater.

In-situ reduction of Cr(VI) to the less toxic and insoluble Cr(III) may be accelerated by electron donor amendment. Chemical electron donors, primarily Fe(II) compounds and sulfides have been successfully used.[2,6] However, substantial

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addition of these reducing agents has some drawbacks, including lowering groundwater pH (for example by $FeSO_4$) or precipitation (sulfides) which may clog the aquifer.[2] Amendment with substrates that would provide electron donors and stimulate indigenous microbial communities is considered a promising approach.[2] Many bacteria, in both pure and mixed cultures, are able to reduce Cr(VI), including nitrate-reducing (NRB), Fe(III)-reducing (FeRB), and sulfate-reducing bacteria (SRB).[7−9] Cr(VI) reduction is observed under various conditions (e.g., aerobic or anaerobic, limited electron donor or acceptor),[10,12,14] and can occur both enzymatically and chemically via the reducing agents Fe(II) and H_2S produced by bacteria.[8,15−18] Cr(VI)-reducing bacteria are frequently isolated from the contaminated Hanford site.[10−13] Recent microcosm experiments with laboratory bioreactors inoculated with groundwater from the Hanford site suggested that lactate amendment stimulated and maintained Cr(VI) reduction even when sulfate was limited.[14,19] However, while previous studies have provided valuable insights into the potential for long-term bioremediation of contaminated groundwater, none have examined Cr(VI)-reducing microbial communities in situ. Critical questions remain, including how the communities change with substrate amendment, and how those changes promote long-term Cr(VI) reduction.

A one-time amendment of polylactate hydrogen-release compound (HRC) at Hanford's 100-H Area reduced groundwater Cr(VI) concentrations for >3.5 years.[5] The HRC amendment enriched groundwater *Pseudomonas*, *Geobacter*, and *Desulfovibrio* species, based on preliminary PhyloChip microarray results.[5] In the current study, the experimental design and HRC amendment are the same as the general groundwater survey focusing on geochemical changes.[5] However, to get a better idea of how the microbial communities changed with HRC amendment and how these changes encouraged continued Cr(VI) reduction, we examined groundwater from this amendment using a comprehensive functional gene microarray (GeoChip 4.0). We hypothesized that (i) HRC amendment would significantly change the functional composition and structure of groundwater microbial communities, (ii) HRC amendment would stimulate functional processes, particularly those related to HRC degradation and reduction of multiple electron acceptors in the contaminated aquifer, and (iii) long-term stimulation of these key functional processes through slow biodegradation of complex HRC would promote long-term Cr(VI) reduction.

MATERIALS AND METHODS

Site Description and HRC Amendment. A detailed description of the site, experimental design, and HRC amendment was previously described[5] and is provided in the Supporting Information (SI). A brief overview is presented below.

HRC (Regenesis, San Clemente, CA) is a heavy (density 1.3 g/cm^3), viscous honey-like liquid. Its main component is glycerol polylactate ($C_{39}H_{56}O_{27}$), a food-grade, complex compound. Upon hydrolysis, glycerol polylactate releases 12 mol of lactate per mole of glycerol. HRC also contains some glycerol, lactate, and a small percentage (1%) of sodium phosphate.[4] To track the fate of HRC after injection, a small amount (10 g) of ^{13}C-labeled lactate was mixed with 18.2 kg HRC.

The study was performed at the U.S. Department of Energy (DOE) 100-H Area in Hanford, WA, along the Cr(VI)-contaminated groundwater pathway from the 100-D Area (point of initial contamination) to the Columbia River (SI Figure S1A). Major contaminants in the groundwater are Cr(VI) (52−84 μg/L), NO_3^- (30−60 mg/L), and SO_4^{-2} (56−86 mg/L), though other metals are also present.[20] To perform field investigations, two ∼18.3 m deep, 15 cm diameter boreholes were cored, forming two wells (699−96−45 (45) and 699−96−44 (44)) ∼5 m apart (SI Figure S1B). The regional groundwater flows from well 45 to well 44. On August 3, 2004, HRC was injected into well 45. The HRC−^{13}C-lactate mixture was preheated to 35 °C and diluted with 15.1 L of distilled water. The injection hose was placed at a depth between 12.8 and 14.0 m (right below the groundwater table), within the high-permeability Hanford sediments (gravel and coarse sand). The hose was filled with 9.5 L of water (as a primer) and then HRC injection was started with a specially designed straddle packer (consisting of two rubber packers separating the injection interval) (SI Figure S1B). After the HRC injection, 17 L of water was injected as a chaser, and on day 2, groundwater was pumped from well 44 to create a direct water flow path between 45 and 44. The hydraulic connection and water flow between these two wells was tested before HRC amendment and monitored periodically (300 and 600 days) after amendment by injecting a potassium bromide solution and pumping for 25 days. Results from borehole water-flux measurements (during ambient and pump testing) showed that practically all water entered the boreholes from the sandy gravel of the Hanford formation, with very low flow from the Ringold formation.

Sampling and Geochemical Analysis. Detailed descriptions of the methods used for geochemical, microbial community, and data analysis are provided in the SI. Briefly, groundwater samples were collected from wells 45 and 44 at four depths (13.1, 14.0, 14.9, and 15.8 m) and seven time points (before HRC injection, and 9, 17, 20, 42, 300, and 390 days after injection). Groundwater was collected by first applying suction and then injecting argon gas (to prevent cross-contamination) to bring water samples to the surface. Groundwater samples were collected from different depths through the open spacing (5 cm long) between inflatable rubber packers using a multilevel sampler. After purging the water samplers, water samples for geochemical analysis (minimum 100 mL) were collected in sterile vials/flasks and first stored on-site at 4 °C and then sent to the analytical laboratory by overnight shipment. For microbial community analysis, groundwater (400 mL) was filtered on site with sterile 8 μm filters to remove large particles, followed by filtering with 0.2 μm filters to collect biomass. The filters were immediately frozen, shipped on dry ice to the laboratory, and stored at −80 °C until DNA extraction.

Geochemical variables, including dissolved organic carbon (DOC), dissolved inorganic carbon,[18] CO_2, ^{13}C, O_2, CH_4, pH, Cr, Fe, Cl, Br, acetate, SO_4^{2-}, NO_3^-, and ^{53}Cr/^{52}Cr ratios of chromium were measured as previously described.[5] Briefly, anions (acetate, NO_3^-, Cl^-, and SO_4^{2-}) were analyzed with an ion chromatograph equipped with an IonPac AS-14 analytical column and an AG-14 guard column (Dionex DX-120, Sunnyvale, CA). Aqueous Fe(II), total Fe, and sulfide were measured colorimetrically using a HACH DR 2000 spectrophotometer (Hach Chemical, Loveland, CO). Cr isotope

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Table 1. Significance of Changes in Key Groundwater Variables and Overall Microbial Community Functional Structure after HRC Amendment Revealed by Three Statistical Tests[a]

	sample groups	MRPP	ANOSIM	Adonis
microbial community	0d vs. 9−42d	**0.001** (0.40)[c]	0.190 (0.18)	**0.002** (2.29)
	9−42d vs. 300−390d	**0.001** (0.40)	0.328 (0.05)	**0.003** (2.40)
	0d vs. 300−390d	**0.019** (0.33)	**0.022** (0.83)	**0.001** (3.52)
groundwater variables[b]	0d vs. 9−42d	**0.002** (0.16)	**0.002** (0.70)	**0.001** (9.67)
	9−42d vs. 300−390d	**0.001** (0.16)	**0.001** (0.54)	**0.001** (9.09)
	0d vs. 300−390d	**0.014** (0.08)	**0.023** (0.88)	**0.001** (21.6)

[a]All three tests are nonparametric multivariate analyses based on dissimilarities between samples in different groups using bray-cutis distance. MRPP, multiple response permutation procedure, a nonparametric procedure that does not depend on assumptions such as normally distributed data or homogeneous variances, but rather depends on the internal variability of the data; ANOSIM, analysis of similarity; Adonis, nonparametric multivariate analysis of variance (MANOVA) with the adonis function. [b]Groundwater variables included microbial biomass (as acridine orange staining direct cell counts), acetate, ^{13}C labeled dissolved inorganic carbons (^{13}C-DIC such as $^{13}CO_2$), phosphate, Cl^-, NO_3^-, NO_2^-, Cr(VI), Fe(II), and SO_4^{-2}. [c]*P* value (statistic). The difference is significant when at least two tests gave *P* values of <0.05 (bold).

composition was analyzed using VG Instruments Sector 54 multicollector TIMS.

Microbial Community DNA Extraction, GeoChip Hybridization, and Statistical Data Analyses. Groundwater DNA was extracted using a BIO101 soil DNA extraction kit (QBiogene, Morgan Irvine, CA). Due to the limited amount of DNA remaining after PhyloChip analysis,[5] only 27 samples were sent to the University of Oklahoma for GeoChip analysis. Upon arrival, DNA quality and quantity were assessed using 260/280 nm and 260/230 nm ratios and the PicoGreen method[21] with a Quant-It PicoGreen kit (Invitrogen, Carlsbad, CA), respectively. DNA (15 ng) was amplified using whole community genome amplification with a modified[22] TempliPhi Kit (GE Healthcare, Piscataway, NJ). Amplified DNA (2 μg) was hybridized with GeoChip 4.0 as previously described.[23] Signal intensities were background-subtracted, and only spots with signal-to-noise ratios ((signal intensity-background intensity)/background standard deviation) >4.5 were considered as positive and used for further analysis.

Various statistical approaches were used to analyze the data as described elsewhere.[24] Briefly, the data were analyzed by (i) microbial diversity indices and significance tests by the Student's *t* test; (ii) hierarchical clustering and detrended correspondence analysis (DCA) to evaluate differences in microbial community composition and structure; (iii) analysis of similarity (ANOSIM), permutational multivariate analysis of variance (Adonis), and multiresponse permutation procedure (MRPP) analysis to determine differences in microbial communities and geochemistry; (iv) canonical correspondence analysis (CCA)[25] to link microbial community structure with groundwater variables; and (v) partial CCA and variation partitioning analysis (VPA) of well distance, sampling depths, and groundwater variables to determine the relative effect of each variable on the community differences.

RESULTS

Changes in Key Groundwater Variables after HRC Amendment. HRC amendment stimulated HRC biodegradation [indicated by increases in groundwater microbial biomass, acetate and ^{13}C-labeled dissolved inorganic carbons (^{13}C-DIC) such as $^{13}CO_2$] and reduction of multiple electron acceptors in the aquifer (e.g., NO_3^-, Cr(VI), Fe(III), SO_4^{2-}). Before HRC amendment, microbial biomass was low (5×10^5 cells/mL on average), the groundwater contained a considerable amount of NO_3^- (30−60 mg/L), SO_4^{2-} (56−86 mg/L), and Cr(VI) (52−84 μg/L), but acetate, ^{13}C-DIC, NO_2^- and H_2S were not detected.[5] After HRC amendment, groundwater biomass increased over 100-fold, and this high level (10^7 cells/mL) was maintained for over a year (SI Figure S2A). Acetate and ^{13}C-DIC were detected at day 9, and high levels of acetate (e.g., 20−120 mg/L) were still detected after 300 days. In addition, sequential reduction of NO_3^-, Cr(VI), Fe(III) and SO_4^- was observed (SI Figure S2B). Cr(VI) was depleted at day 17, almost concurrently with the substantial NO_3^- reduction. Low groundwater Cr(VI) concentrations (<22 μg/L) were then maintained. Higher Cr(VI) and NO_3^- concentrations were observed in the upgradient injection well (45) at 9−17 days, likely due to mixing with a regional groundwater flow containing Cr(VI) and NO_3^- during the post-HRC injection pumping. Extensive Fe(III) and SO_4^- reduction was observed later, and substantially decreased SO_4^{-2} and high groundwater Fe(II) concentrations (~10 mg/L) were detected after 300 days. Three complementary nonparametric multivariate statistical tests (MRPP, ANOSIM, and Adonis) of groundwater biomass, acetate, ^{13}C-DIC, PO_4^{-3}, Cl^-, NO_3^-, NO_2^-, Cr(VI), Fe(II), and SO_4^{-2} concentrations suggested that significant ($P < 0.01$ or 0.05) HRC biodegradation and electron acceptor reduction occurred after HRC amendment, and that high redox activities were maintained for over a year in the aquifer (Table 1).

Shifts of Overall Groundwater Microbial Communities. Several statistical analyses of all detected functional genes suggested that HRC amendment stimulated sequential shifts in the overall functional composition and structure of groundwater microbial communities. First, DCA analysis separated all samples into three major clusters: before HRC amendment [day 0 (0d)], early post-HRC amendment [days 9−42 (9−42d)], and late post-HRC amendment [days 300−390 (300−390d)] (Figure 1A). Groupings of these samples were largely consistent with the measured differences in HRC biodegradation and electron acceptor reduction. On day 0, all of the samples clustered together closely. By day 9 after HRC amendment, the microbial communities had shown an obvious shift (along the second axis) in composition and structure. While there was a fair amount of variation between samples at different time points, the communities remained in this general cluster through day 42. During this period (9−42d), extensive HRC degradation and NO_3^- and Cr(VI) reduction were detected (SI Figure S2). By day 300, when extensive Fe(III) and SO_4^{-2} reduction was also observed, the communities showed a further shift (along the first axis) in functional composition and structure. The communities remained within

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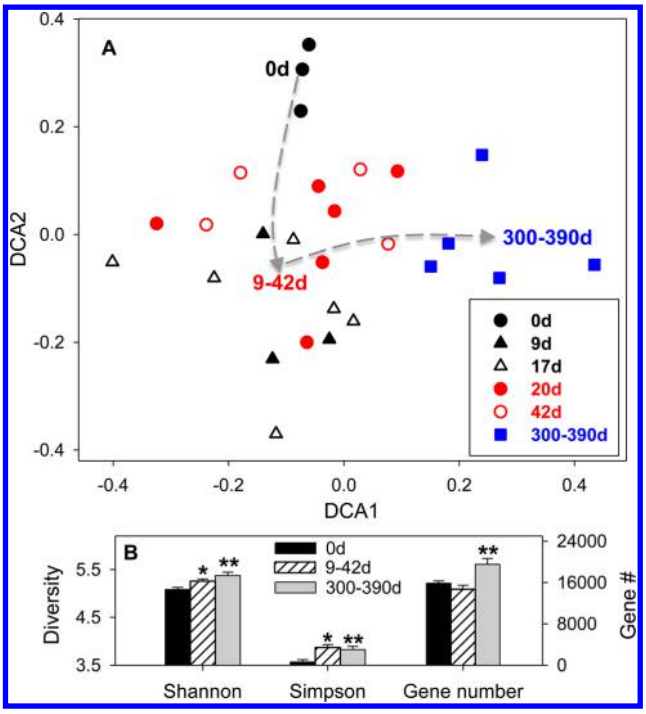

Figure 1. (A) Detrended correspondence analysis (DCA) of all detected functional genes, showing that the groundwater microbial communities underwent three succession stages, 0d (before HRC amendment), 9−42d after HRC amendment, and 300−390d after HRC amendment. (B) Average functional diversity and total gene number detected at these succession stages. Significance (**P < 0.05,*P < 0.10) was tested between 0d and either 9−42d or 300−390d using the Student's t test. All data are presented as mean ± SE. The numbers of samples were three for 0d, 19 for 9−42d, and five for 300−390d.

this final cluster for the remainder of the study (390 days). Second, Shannon−Weiner (H') and Simpson's ($1/D$) diversity indices marginally increased (P < 0.10) at 9−42d and significantly increased (P < 0.05) at 300−390d, when the detected functional gene number also increased (P < 0.05) (Figure 1B). Also, three dissimilarity tests (MRPP, ANOSIM and Adonis) suggested that the functional composition and structure of groundwater microbial communities underwent two major shifts after HRC amendment (P < 0.01 for MRPP and Adonis), from 0d to 9−42d and then from 9−42d to 300−390d, resulting in significantly modified communities at 300−390d (P < 0.05 for all three tests when compared to 0d) (Table 1). As such, for all further statistical analyses, the samples were grouped into three sets, 0d, 9−42d, and 300−390d.

Changes of Key Functional Genes Involved in Important Microbial Processes. To assess potential changes in important microbial processes after HRC amendment, various key functional genes (SI Table S1) were examined based on their abundance (signal intensity) in the groundwater microbial communities.

(i). Carbon (C) Cycling Genes (HRC Biodegradation). The abundance of *fhs* genes encoding formyltetrahydrofolate synthetase, which is involved in acetate oxidation, significantly (P < 0.05) increased after HRC amendment (Figure 2), suggesting a stimulation of the final step of HRC degradation and acetate, H_2, and CO_2 production. Clustering analysis of *fhs* genes enriched at either 42d (SI Figure S3A) or 300−390d (SI Figure S3B) after HRC amendment and those detected before the amendment indicated that the HRC stimulated samples clustered together and were well separated from the 0d samples. Although some genes (e.g., *fhs* from *Rubrobacter xylanophilus*) were common in all samples, most of the detected *fhs* genes showed increased abundance after HRC amendment. Examples of enriched *fhs* genes included those similar to *Syntrophus aciditrophicus*, known to degrade fatty acids,[26] and *Caldicellulosiruptor saccharolyticus*, able to degrade polymeric carbohydrates to acetate, H_2, and CO_2.[27]

Since some of the HRC-enriched biomass could decay at later stages of the experiment, a variety of genes potentially associated with biomass decomposition were also examined and results suggest that this decay may be providing an additional

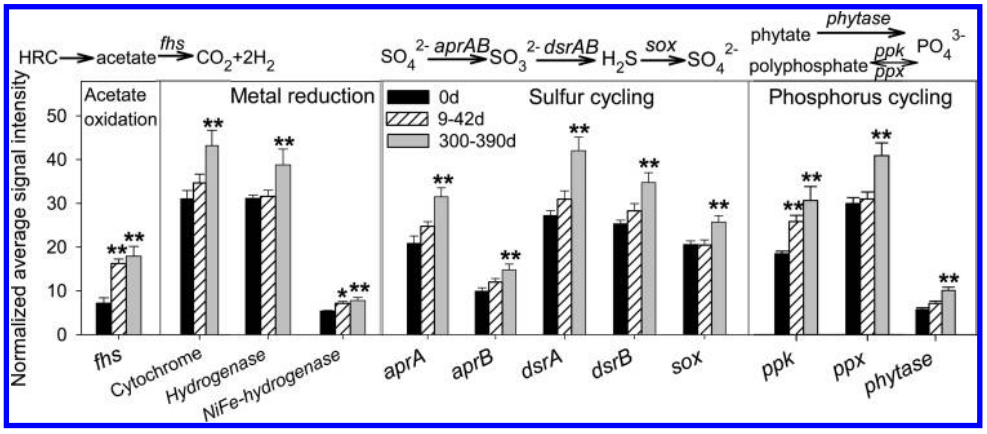

Figure 2. Normalized signal intensity of detected key genes involved in acetate oxidation, metal reduction, sulfur cycling, and phosphorus cycling. The transformations catalyzed by these genes are shown: *fhs* encoding formyltetrahydrofolate synthetase for acetate oxidation; *cytochrome* encoding cytochrome c_3 and *hydrogenase* encoding hydrogenase for metal reduction; *aprA/B* encoding adenosine-5′-phosphosulfate reductase subunits for dissimilatory sulfate reduction, *dsrA/B* encoding dissimilatory sulfite reductase subunits for sulfite reduction, and *sox* encoding sulfur-oxidizing enzyme for H_2S reoxidation; *ppk* encoding polyphosphate kinase responsible for polyphosphate synthesis, *ppx* encoding exopolyphosphatase responsible for polyphosphate hydrolysis, and *phytase* encoding phytate phosphohydrolase for phytate hydrolysis. Signal intensities are the sum of detected individual gene sequences for each gene, averaged among three samples at 0d (before HRC amendment), among 19 samples at 9−42d after HRC amendment, and among five samples at 300−390d after HRC amendment. All data are presented as mean ± SE **P < 0.05,*P < 0.10, Student's t test.

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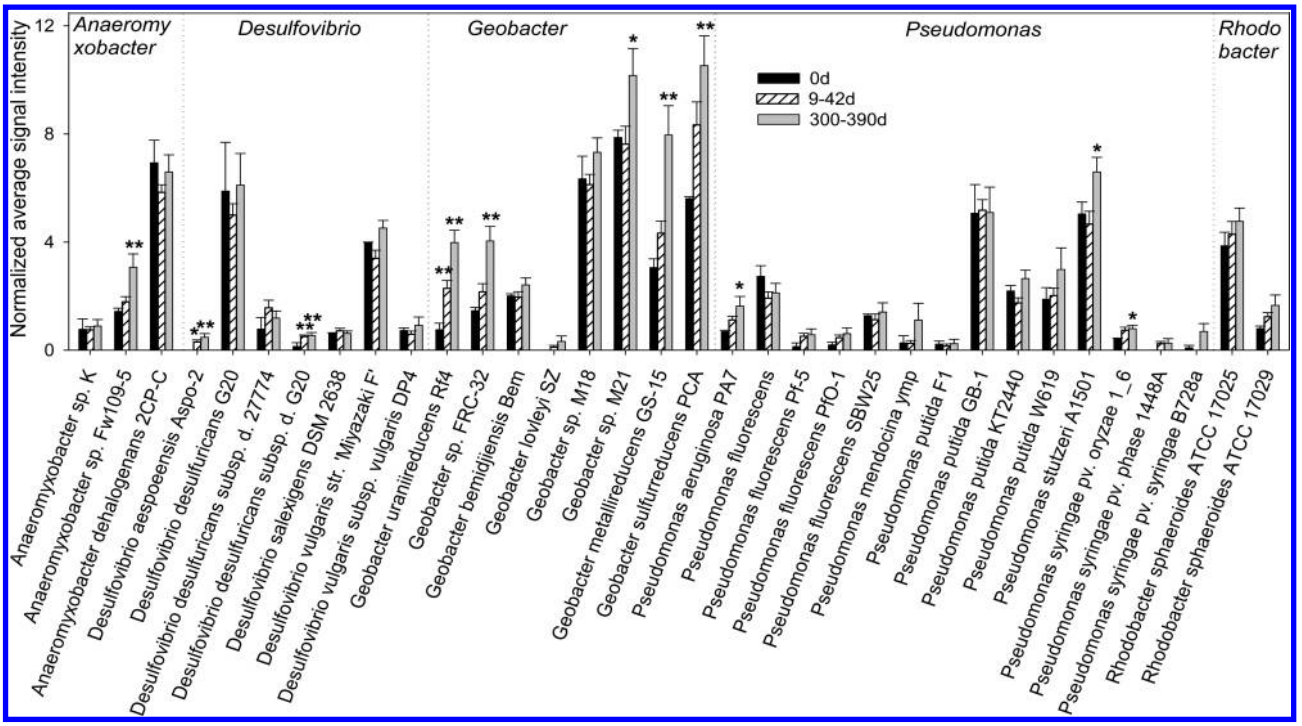

Figure 3. Normalized signal intensity of the detected cytochrome c_3 genes derived from various organisms at 9−42d and 300−390d after HRC amendment compared to the preamendment condition (0d). The genes are presented in the order of the affiliated genera: *Anaeromyxobacter*, *Desulfovibrio*, *Geobacter*, *Pseudomonas*, and *Phodobacter*. The signal intensities are the sum of detected individual gene sequences in each organism, averaged among 3 samples at 0d (before HRC amendment), among 19 samples at 9−42d after HRC amendment, and among 5 samples at 300−390d after HRC amendment. All data are presented as mean ± SE $^{**}P < 0.05$,$^{*}P < 0.10$, Student's *t* test.

source of C and other nutrients. Genes involved in bacteriophage functions (particularly replication) increased at 9−42d as microbial biomass increased, and the gene abundance continued to increase at 300−390d (SI Figure S4). Also, of the 33 organic C (e.g., starch, hemicellulose, cellulose) degradation genes detected, 14 (42%) (e.g., *glucoamylase*, *xylanase*) showed significantly ($P < 0.05$) increased abundance at 300−390d (SI Figure S5). In addition, genes involved in biodegradation of organic nitrogen (N) (e.g., *ureC* encoding urease for amino acid biodegradation) and organic phosphorus (P) (*phytase* encoding phytate phosphohydrolase for phytate hydrolysis) compounds significantly ($P < 0.05$) increased at 300−390d (Figure 2 and SI Figure S6B).

(ii). N Cycling Genes (Nitrate Reduction). The abundance of most (75%) of the genes involved in nitrate reduction significantly ($P < 0.05$) increased at 9−42d after HRC amendment (SI Figure S6A), and a majority of these enriched genes (denitrification and dissimilatory nitrate reduction) remained elevated through 300−390d (SI Figure S6B and C). *nirA* encoding nitrite reductase for assimilatory nitrate reduction marginally ($P < 0.10$) increased during the period of extensive microbial growth (9−42d). Denitrification genes were also stimulated, with increased abundances of *narG* encoding nitrate reductase, *nirS* encoding nitrite reductase, and *nosZ* encoding N_2O reductase. In addition, genes involved in dissimilatory nitrate reduction were enriched, including *napA* encoding nitrate reductase and *nrfA* encoding nitrite reductase. Dissimilatory nitrate reduction genes from NRB, FeRB, and SRB (e.g., *Pseudomonas*, *Geobacter uraniumreducens*, *G. bemidjiensis*, *Desulfovibrio desulfuricans*, *Desulfitobacterium hafniense*) were stimulated as well as some denitrification genes from *Pseudomonas* spp. (data not shown).

(iii). S Cycling Genes (Sulfate Reduction). Sulfate in the groundwater flow is another significant electron acceptor. HRC amendment also stimulated key genes involved in sulfate reduction. The abundance of *aprAB*, encoding dissimilatory adenosine-5′-phospho-sulfate reductase, and *dsrAB*, encoding dissimilatory sulfite reductase, both of which are involved in dissimilatory sulfate reduction, significantly ($P < 0.05$) increased at 300−390d (Figure 2). More specifically, a total of 440 *dsrA* and 313 *dsrB* genes were detected. Although a large portion (~80%) of the enriched *dsrAB* genes were from uncultured SRB, analysis of the genes derived from known species suggested that various SRB were significantly ($P < 0.05$) enriched after HRC amendment, including those genera with members known to be capable of Cr(VI) reduction (e.g., *Desulfovibrio vulgaris*, *Desulfotomaculum reducen* MI-1)[8,18] (SI Figure S7 and S8). SRB known to degrade short-chain fatty acids (SCFAs) (e.g., *Desulfobacterium*, *Syntrophobacter*)[28−30] were also stimulated.

(iv). Genes Involved in Metal Reduction. Genes involved in H_2 production and electron transport were examined since HRC biodegradation is expected to produce H_2[5,31] and some hydrogenases and cytochromes c_3 are involved in electron transport from H_2 to Cr(VI) and other metals (e.g., Fe(III), U(VI)).[15,18] The GeoChip contained 364 probes specifically designed to detect cytochrome c_3 genes from important U(VI)-reducing *Geobacter*, *Anaeromyxobacter*, and *Desulfovibrio* species. A total of 188 cytochrome c_3 genes were detected, and the gene abundance significantly ($P < 0.05$) increased at 300−390d compared to 0d (Figure 2). Although the abundance of some genes (e.g., those from *Geobacter uraniireducens* Rf4 and *Desulfovibrio desulfuricans* subsp. desulfuricans G20) increased at 9−42d, most of the enriched cytochrome c_3 genes increased

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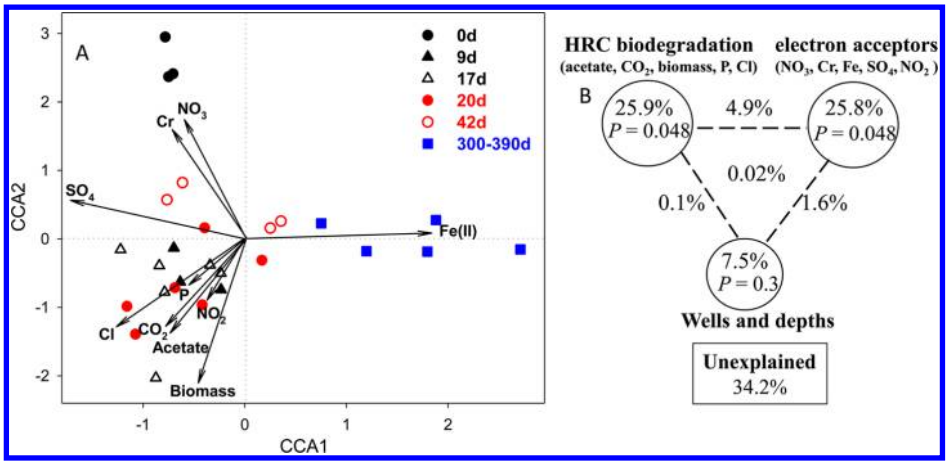

Figure 4. (A) Canonical correspondence analysis (CCA) shows significant (P = 0.005) correlations between signal intensities of all detected functional genes (symbols) and environmental variables (arrows). Environmental variables were chosen based on significance calculated from individual CCA results and variance inflation factors (VIFs < 20) calculated during CCA. (B) Variation partitioning based on CCA. The HRC biodegradation variables included microbial biomass (as direct cell counts), acetate, $^{13}CO_2$ (as ^{13}C-labeled dissolved inorganic carbons), phosphate, and Cl^-, and electron acceptor variables included NO_3^-, NO_2^-, Cr(VI), Fe(II), and SO_4^{-2}. Wells and depths included two wells (699−96−44 and 699−96−45) and four sampling depths (13.1, 14.0, 14.9, and 15.8 m).

later (Figure 3). Particular cytochrome c_3 genes enriched (P < 0.05) at 300−390d included those from *Geobacter* subsurface clade 1 species (e.g., *G. uranireducens* Rf4, *G.* sp. FRC-32, *G.* sp. M21), known to be predominant in U(VI)- and Fe(III)-reducing environments,[32] whereas genes from *Pseudomonas* spp., *Desulfovibrio* spp., and *Anaeromyxobacter* spp. were also enriched. Similarly, the total abundance of hydrogenase genes significantly (P < 0.05) increased at 300−390d (Figure 2), and the genes derived from these *Geobacter*, *Anaeromyxobacter*, and *Desulfovibrio* species were also enriched (SI Figures S9 and S10).

(v). Other Genes (C and N Fixation, Cr(VI) Resistance). It was also noted that the abundance of genes involved in CO_2 fixation (e.g., propionyl-CoA carboxylase), N_2 fixation (*nifH* encoding nitrogenase), and polyphosphate hydrolysis (*ppx* encoding exopolyphosphatase) significantly (P < 0.05) increased at later stages of this experiment (300−390d) (Figure 2, SI Figure S5 and S6). Further analysis suggested that many of the enriched *nifH* genes were also from *Geobacter* (e.g., *G. uraniumreducens* Rf4, *G.* sp. FRC-32, *G. bemidjiensis*, *G.* sp. M21, *G. lovleyi* SZ), *Anaeromyxobacter* (e.g., *A. dehalogenans* 2CP-C), and *Desulfovibrio* (e.g., *D. vulgaris* "Miyazaki F") (SI Figure S11).

Indigenous microbial communities at this site would be expected to be resistant to Cr(VI), and HRC amendment would likely stimulate resistant populations. The abundance of *chrA* genes, encoding Cr(VI) efflux transporters for Cr(VI) resistance, significantly (P < 0.05) increased (SI Figure S12). Many of the enriched *chrA* genes were also from *Geobacter* (e.g., *G. uraniumreducens* Rf4, *G.* sp. FRC-32, *G. bemidjiensis*, *G.* sp. M21, *G. lovleyi* SZ), *Anaeromyxobacter* (e.g., *A. dehalogenans* 2CP-C), and *Desulfovibrio* (e.g., *D. vulgaris* "Miyazaki F") (SI Figure S11). In addition to Cr(VI), this site is contaminated with other metals (e.g., As, Cd, Pb), though at low concentrations (0.8−20.1 mg/kg).[20] A large number (3728) of genes involved in the resistance to other metals (e.g., As, Cd, Co, Ni, Pb, Hg, Se, Ag, Te) were detected as well, and some of them increased (P < 0.05) after HRC amendment (SI Figure S12).

HRC Biodegradation and Electron Acceptor Reduction as Important Factors Affecting Community Functional Composition and Structure. CCA analysis was performed to identify key variables affecting the composition and structure of groundwater microbial communities. On the basis of variation inflation factors (VIFs < 20), 10 groundwater variables were identified: five (microbial biomass, acetate, ^{13}C-DIC, PO_4^{-3} and Cl^-) related to HRC biodegradation, and the remainder related to electron acceptors (NO_3^-, Cr(VI), Fe(II), SO_4^{-2}, and NO_2^-). The shifts in the functional composition and structure of groundwater microbial communities after HRC amendment were significantly (P = 0.005) correlated with changes in the groundwater concentrations of these variables (Figure 4A). Specifically, before HRC amendment, microbial communities were most significantly affected by high groundwater Cr(VI) and NO_3^- concentrations; at 9−42d, the communities were most significantly correlated with microbial growth, HRC biodegradation, and Cr(VI) and NO_3^- reduction; and at 300−390d, the communities were most significantly correlated with SO_4^{-2} and Fe(III) reduction. The small angles between the vectors indicated a strong correlation between CO_2 and acetate production, between Cr(VI) and NO_3^- reduction, and between Fe(III) and SO_4^{-2} reduction.

Variation partitioning analysis[33] was performed to determine the individual contributions of HRC biodegradation and electron acceptor reduction to the community shifts. These two variable groups were equally important and significantly (P = 0.048) explained a considerable portion (52%) of the total community changes after HRC amendment (Figure 4B), whereas factors like well location and sampling depth only explained a small portion (7.5%, P = 0.3) of the community changes. About 34% of the community changes remained unexplained, which could be attributed to changes in other factors important to this experiment, such as H_2, butyrate, and Mn(II), which were not measured in this study.

DISCUSSION

Bioremediation has been successfully used for U(VI)-contaminated groundwater at other DOE sites. Frequent (continuous, daily, or weekly) injections of fast-degrading

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substrates (e.g., acetate, ethanol, lactate) reduced groundwater U(VI) concentrations at the Oak Ridge and Rifle sites, while a one-time amendment of emulsified vegetable oil (EVO) supported U(VI) reduction for one year at the fast-flowing Oak Ridge aquifer.[34−39] Microbial communities were extensively investigated at both of these contaminated sites. Results suggest that microorganisms capable of redox reactions (e.g., substrate degraders, NRB, FeRB, SRB) are present in contaminated aquifers and that stimulation of these microorganisms by amending hydrogen-release substrates (e.g., ethanol, EVO) may promote long-term U(VI) reduction.[40−42] The prolonged effectiveness of complex EVO is largely due to its high energy density, slow biodegradation, and retarded flow in groundwater systems,[43] allowing an extended supply of electron donors and C sources with a one-time amendment.

Using a comprehensive functional gene microarray, this study examined Cr(VI)-reducing microbial communities after a one-time HRC amendment at the Hanford site, and linked the functional composition and structure of the microbial communities with key groundwater variables to understand how changes to the community promote long-term (3.5 years) Cr(VI) reduction. The results showed that the overall communities underwent sequential shifts after HRC amendment, and key functional genes involved in various redox processes in particular were stimulated. The overall community dynamics was significantly correlated with changes in groundwater concentrations of microbial biomass, acetate, NO_3^-, Cr(VI), Fe(II), and SO_4^{2-}. This study provides new insights into our understanding of the microbial communities responsible for Cr(VI) reduction in situ and identifies some key functional processes/genes (e.g., acetate oxidation, denitrification, dissimilatory nitrate reduction, metal reduction, sulfate reduction). Our results suggest that long-term stimulation of these key functional processes/genes may sustain Cr(VI) reduction. The time-series dynamics of these processes/genes could play a role in maintaining Cr(VI) reduction in the Hanford aquifer. This information may be useful for bioremediation of other contaminated aquifers.

We observed sequential shifts in the functional composition and structure of groundwater microbial communities after HRC amendment, likely due to the stepwise HRC degradation and electron acceptor reduction.[5,31] After amendment, HRC first slowly hydrolyzes into glycerol and lactate. The glycerol and lactate are then degraded, producing H_2, CO_2, acetate, and other SCFAs (e.g., butyrate). These degradation intermediates/products serve as electron donors and C sources and stimulate sequential reduction of electron acceptors in the aquifer (e.g., NO_3^-, Cr(VI), Fe(III), SO_4^{2-}). Since all these functional processes involve an array of functional genes, sequential shifts in the overall functional composition and structure of groundwater microbial communities are expected. These results agree in general with previous studies reporting sequential shifts in the phylogenetic composition and structure of groundwater microbial communities after EVO amendment.[40] Thus, we also hypothesized that HRC amendment would stimulate key functional genes involved in HRC degradation and electron acceptor reduction. Few studies have examined the genes involved in functional processes associated with substrate degradation. Proteomics did detect an increase in fatty acid-degrading enzymes (e.g., flavoproteins, thiolase) in U(VI)-contaminated groundwater, 4 days after EVO amendment.[41] In this study, we observed increased abundance of key functional genes involved in acetate oxidation, bacteriophages and organic C, N, and P biodegradation, suggesting a stimulation of metabolic potential associated with HRC biodegradation and biomass decomposition. Decaying microbial biomass could provide C and energy sources at later stages of HRC amendment.[5]

Competition for electrons has been observed between other electron acceptors (e.g., NO_3^-) and U(VI). With ethanol injection, NO_3^- was reduced before Fe(III) and U(VI).[44] After EVO amendment, reduction in the order of NO_3^-, Mn(IV), Fe(III), U(VI) and SO_4^{2-} was observed.[40] Time-series dynamics was also observed for some key functional genes involved in electron acceptor reduction. Proteomic analysis showed that NosZ was highly abundant 4 days after EVO amendment, but that the abundance of cytochromes c_3 was still fairly low at this early time point.[41] Another study reported that the abundance of cytochrome c_3 genes decreased and dsrAB genes increased when subsurface redox conditions shifted from Fe(III) reduction to SO_4^{2-} reduction after acetate injection.[37] Laboratory experiments with a groundwater isolate from the contaminated Hanford site also showed that lactate addition increased the transcript copy number of *narG* and *nirS* during simultaneous denitrification and Cr(VI) reduction.[10] In the current study, while sequential reduction of multiple electron acceptors that typically coexist in contaminated aquifers is unsurprising, the particular order of reduction (NO_3^-, Cr(VI), Fe(III), and SO_4^{2-}) was different, most likely because of the redox potential of Cr(VI) (1.33 V) vs U(VI) (−0.042 to 0.086 V).[45] Also, we observed an increased abundance of a variety of key functional genes involved in denitrification (e.g., *narG*, *nirS*, *nosZ*), dissimilatory nitrate reduction (*napA*, *nrfA*), metal reduction (hydrogenase and cytochrome c_3 genes), and SO_4^{2-} reduction (*AprAB*, *dsrAB*), suggesting a diversity of functional processes and key functional genes are involved in subsurface redox reactions after substrate amendment. Further, statistical analyses suggested a time-series dynamic to microbial community, functional process, and key gene changes. The first major community shift (0d to 9−42d) was characterized by significant increases of some key functional genes (e.g., acetate oxidation, denitrification, dissimilatory nitrate reduction), HRC biodegradation and NO_3^- and Cr(VI) reduction. A second major community shift followed (9−42d to 300−390d) and was characterized by an overall increase of most key functional genes and Fe(III) and SO_4^{2-} reduction while NO_3^- and Cr(VI) reduction continued. Overall, our results support the second hypothesis, that HRC amendment stimulated functional processes involved in HRC biodegradation and electron acceptor reduction.

With a one-time amendment of slowly degrading, complex substrates like EVO and HRC, the most important question for bioremediation applications is whether the shifts in microbial community composition and structure would enhance long-term metal (e.g., U(VI), Cr(VI)) reduction. Phylogenetic analyses have indicated that enrichment of NRB, FeRB, and SRB may sustain U(VI) reduction. A long-term stimulation of FeRB and SRB, like *Geobacter*, *Anaeromyxobacter*, *Desulfovibrio*, and *Desulfotomaculum*, is associated with U(VI) reduction of >1 year, while stimulation of NRB encourages the removal of excess competing NO_3^-.[40,42,46,47] It has been suggested that after Fe(III) is depleted during initial U(VI) reduction, SRB could continue to grow using the SO_4^{2-} continuously entering the environment via groundwater flow and sustain U(VI) reduction if a hydrogen-release substrate is injected.[8,35,48,49] Enrichment of *Pseudomonas*, *Geobacter*, and *Desulfovibrio* was

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observed after HRC amendment by PhyloChip analysis.[5] In addition to phylogenetic genes (16S rRNA), some functional genes (e.g., *dsrAB*, hydrogenase genes) have been used as biomarkers to track associated microbial populations.[50−56] In the current study, the increased abundance of multiple functional genes suggested that diverse NRB, FeRB, and SRB were enriched after HRC amendment, including many *Geobacter* subsurface clade 1 species (e.g., *G. uraniumreducens* Rf4, *G.* sp. FRC-32, *G.* sp. M21), which are known to be predominant in various U(VI)- and Fe(III)-reducing environments,[32] and species of *Pseudomonas*, *Desulfovibrio*, *Desulfotomaculum*, *Desulfitobacterium*, and *Anaeromyxobacter*. Further, the enriched functional genes derived from these microorganisms were diverse, indicating that these NRB, FeRB, and SRB have the potential to utilize multiple electron acceptors (e.g., NO_3^-, Fe(III), SO_4^{2-}), fix N_2, and be resistant to Cr(VI). Overall, our results suggest that the enriched NRB, FeRB, and SRB are phylogenetically diverse, physiologically versatile, and ecologically robust in the aquifer and play important roles in prolonging Cr(VI) reduction. Such observations provide a possible mechanistic explanation for the predominance of NRB, FeRB, and SRB in various metal (e.g., Fe(III), U(VI), Cr(VI)) reducing environments.[32,57]

Lastly, the stepwise enrichment and activity of NRB, FeRB, and SRB may contribute to the long-term Cr(VI) reduction. Different from U(VI) reduction, which generally occurs after nitrate is reduced,[44] in the current study, Cr(VI) reduction occurred concurrently with nitrate reduction. Denitrifiers capable of Cr(VI) reduction are frequently reported[12] and isolates (e.g., *Pseudomonas stutzeri* RCH2) from the Hanford site have been shown to reduce Cr(VI) rapidly under denitrifying or aerobic conditions.[10] In addition, Cr(VI) reduction was dramatically accelerated when nitrate (or O_2) reduction occurred at the same time and nitrate and Cr(VI) reduction rates were highly correlated.[10] These studies, along with our findings, suggest that simultaneous nitrate and Cr(VI) reduction could occur in the field at a much higher redox potential than for U(VI). Initial Cr(VI) reduction could also facilitate growth of some FeRB and SRB that have fairly low tolerances for Cr(VI).[58,59] After the redox potential decreased, substantial growth of FeRB and SRB could play an important role in the long term Cr(VI) reduction, both enzymatically and chemically via the bacterially produced Fe(II) and H_2S.[18,58] Substantial amounts of Fe(II) (0.2 mM) and H_2S (0.8 mM) were detected in the groundwater at 300−390d.

In summary, this study identified some key functional genes/populations involved in functional processes important to Cr(VI) reduction, and mechanisms by which continued reduction was maintained. While changes in gene abundance suggest increases or decreases in population, which can also be used to infer activity, to truly examine activity, measurement at the RNA or protein level should be conducted to further test the hypotheses developed in this study. Additionally, monitoring more samples and wells for longer periods may provide more insight into the community succession during this long-term Cr(VI) reduction. This study, examining Cr(VI)-reducing communities in situ, improves our understanding of microbial communities, functional processes, and the dynamics at play in maintaining long-term Cr(VI) reduction at the Hanford site. While additional studies of sediment microbial communities are needed, the knowledge gained from examining the groundwater microbial community will help us further improve bioremediation designs for long-term bioremediation at other contaminated sites.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b00024.

A detailed description of the site, experimental design, and HRC amendment; 12 figures, and a table (PDF)

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Notes

The authors declare no competing financial interest.

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