Effect of Bare and Coated Nanoscale Zerovalent Iron on tceA and vcrA Gene Expression in Dehalococcoides spp.

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Nanoscale zerovalent iron (NZVI) can be used to dechlorinate trichloroethylene (TCE) in contaminated aquifers. Dehalococcoides spp. is the only microbial genus known to dechlorinate TCE to ethene as a respiratory process. However, little is known about how NZVI affects the expression of genes coding for reductive dechlorination. We examined a high-rate TCE-dechlorinating mixed culture which contains organisms similar to known Dehalococcoides to study the effects of NZVI on the expression of two model genes coding for reductive dehalogenases (tceA and vcrA). A novel pretreatment approach, relying on magnetic separation of NZVI prior to reverse transcription qPCR (to avoid RNA adsorption by NZVI), was developed and used with relative quantification (relative to 16S rRNA as endogenous housekeeping gene) to quantify reductive dehalogenase gene expression. Both tceA and vcrA were significantly down-regulated (97- and 137-fold, respectively) relative to baseline (time 0) conditions after 72-h exposure to chlorinated ethenes (0.12 ± 0.03 mg/L cis-DCE, 0.69 ± 0.11 mg/L t-DCE, and 0.54 ± 0.16 mg/L VC) and bare-NZVI (1 g-NZVI/L). However, coating NZVI with an olefin maleic acid copolymer (a common approach to enhance its mobility in aquifers) overcame this significant inhibitory effect, and both tceA and vcrA were up-regulated (3.0- and 3.5-fold, respectively) after 48-h exposure. Thus, NZVI coating might enhance the expression of dechlorinating genes and the concurrent or sequential participation of Dehalococcoides spp. in the remediation process.

Introduction

Nanoscale zerovalent iron (NZVI) may be used as a reducing agent for in situ remediation of chlorinated ethene contaminated sites (1–4). NZVI has a relatively high specific surface area and reactivity, and may be deployed in situ by slurry injection (5). Various types of NZVI, including surface-coated and bimetallic NZVI (1, 3, 6–8), have been extensively studied over the past decade and used to promote reductive dechlorination of chlorinated-solvent plumes (1) and DNAPL source zones (3, 4). Although NZVI is an effective bulk reductant for dechlorination, recent findings suggest that its use in situ may have unintended and detrimental environmental impacts. For example, bare (uncoated) NZVI can be toxic to bacteria (9–11). The mode of this toxicity is unknown but some evidence points toward the generation of reactive oxygen species by NZVI (9, 10).

Recent studies demonstrate that the bacterial toxicity of NZVI is mitigated by coating the particles with engineered polymer or natural organic matter (11, 12). Li et al. (11) suggested that direct contact between bacteria and NZVI may be important for toxicity and that coatings on NZVI limit contact with the cells via electrostatic and/or electrostatic repulsion. The emerging complexity of NZVI toxicity underscores the need to advance a fundamental understanding of how NZVI interacts with bacteria to better predict the potential for detrimental environmental impacts and perhaps optimize NZVI-based strategies for chlorinated ethenes remediation. Dehalococcoides is the only known genus that completely dechlorinates trichloroethylene (TCE) to ethene, and members of this phylotype are commonly found in subsurface environments contaminated with chlorinated ethenes (13). These indigenous bacteria are important during stimulated bioremediation and natural attenuation of chlorinated solvents (14). Additionally, Dehalococcoides may serve as polishing agents following partial NZVI-based reductive dechlorination in a permeable reactive barrier or reactive zones created by NZVI injection. Previous work in our laboratory demonstrated that NZVI stimulated methanogenic activity (15, 16) while inhibiting biological dechlorination in a mixed culture containing Dehalococcoides spp.

The inhibition of dechlorination suggests that Dehalococcoides spp. may be particularly sensitive to NZVI. However, the mechanism of inhibition of dechlorination has not been explored further and it is unknown whether bacterial toxicity may be associated with inhibition of dechlorinating gene expression or associated enzyme activity. Dehalococcoides spp. harbor reductive dehalogenase (RDase) genes, such as tceA, vcrA, and bvcA, which are responsible for their dechlorinating activity (17, 18). The product of the tceA gene in Dehalococcoides strains 195 and FL2 is thought to be responsible for the transformation of TCE to cis-dichloroethylene (cis-DCE) and vinyl chloride (VC) and cometabolism of VC to ethene (19–21). The vcrA gene in Dehalococcoides strains VS and GT codes for the enzyme that catalyzes the reduction of DCE and VC to ethene (22, 23). These genes and their mRNA transcripts are important biomarkers for evaluating in situ reductive dechlorination as well as the physiological state of the Dehalococcoides populations (18, 24, 25), and may also serve as important biomarkers to assess the impact of NZVI on dechlorinating activity in mixed cultures containing Dehalococcoides. This paper describes the effect of NZVI on the transcription of two functional genes that code for the dechlorination activity of Dehalococcoides spp. Employing a novel pretreatment approach based on magnetic separation of NZVI from cells (to avoid RNA adsorption by NZVI and associated interference from dissolved iron on qPCR), we quantified relative transcription levels of tceA and vcrA genes in a mixed dechlorinating culture in the presence and absence of NZVI.

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Parallel studies with coated and bare NZVI provide insight into the nature of the inhibitory mechanisms and the potential for coatings to reduce or eliminate the inhibitory effect of NZVI on biological dechlorination activity.

Materials and Methods

Chemicals and NZVI. Trichloroethylene, cis-dichloroethene (cis-DCE), trans-dichloroethene (trans-DCE), and vinyl chloride (VC) were purchased from Sigma-Aldrich (St. Louis, MO). Molecular biology grade β-mercaptoethanol was purchased from Research Organics (Cleveland, OH). NZVI particles were obtained from Toda Kogyo Corporation (Onoda, Japan). The physical and chemical properties of this NZVI can be found in Liu et al., 2005 (8). Briefly, the NZVI suspension consisted of irregularly shaped particles ranging in size from 5 to 70 nm with a median radius of 20 nm. Its surface is negatively charged. Coated NZVI (coated by a negatively charged polyelectrolyte—olefin maleic acid copolymer, MW 16,000 g/mol) was also supplied by Toda Kogyo Corporation. Olefin maleic acid is not toxic to bacteria and is a representative of the carboxylated organic macromolecules which are often used to prevent NZVI aggregation during emplacement (26–28). The slurry was prepared as described earlier (29).

Cultures and Medium Preparation. A Dehalococcoides-containing (∼10^6 gene copies/mL) TCE enrichment culture was developed from an anaerobic methanogenic consortium that had been functionally stable in our laboratory for more than 10 years (30, 31). An inoculum of this culture was created by anaerobically transferring 800 mL to a 1-L serum bottle with a Teflon-lined stopper and aluminum crimp seal capped port on the side to add TCE as electron acceptor.

Analytical Procedures. TCE, cis-DCE, t-DCE, and VC were analyzed using a packed column (6 ft. × 1/8 in. o.d. 60/80 carboxapak B/1% SP-1000, Supelco, Bellefonte, PA) on a GC (HP5890, Ramsey, MN) equipped with a flame ionized detector (FID) as described previously (15).

Transmission Electron Microscopy (TEM). E. coli (ATCC strain KW12) was grown in LB Miller broth medium at 37 °C for 12 h. The bacteria were harvested by centrifugation at 5000g for 1 min. E. coli stock was prepared by resuspending bacteria pellets in 10 mL of 2 mM sodium bicarbonate. NZVI-coated-NZVI (100 mg-NZVI/L) were added to the bacteria stock and incubated for 1 h. To examine the interaction of bacteria with coated or bare NZVI, a 10-µL aliquot of the NZVI—bacteria mixture was plated on 400-mesh copper grids (ultrathin carbon type-A, Ted Pella, Redding, CA) and dried overnight. The NZVI—bacteria samples were examined by TEM performed with a JEOL 1230 operated at 120 kV (JEOL, Tokyo, Japan).

NZVI and Coated-NZVI Exposure Experiment. The Dehalococcoides-containing culture was activated by feeding with TCE (228 mg/L, dissolved in methanol—electron donor) for three cycles. Residual chlorinated ethenes present at the beginning of the experiment were cis-DCE (0.12 ± 0.03 mg/L), t-DCE (0.69 ± 0.11 mg/L), and VC (0.54 ± 0.16 mg/L). TCE was not added during these experiments to avoid confounding effects on RDase gene expression associated with differences in TCE concentrations between treatments, resulting from differences in biological, abiotic, and combined dechlorination rates.

After the culture was activated, two treatments were set up in triplicate containing 100 mL of culture per serum bottle (Wheaton, NJ, USA) with/without NZVI (1 g/L, Fe^0 ∼40%). This concentration is within the range (1–10 g/L) commonly used when NZVI is injected into the subsurface for remediation purposes (32). The experiment with coated NZVI was set up in the same manner. Specifically, 833 µL of coated-NZVI solution (120 g-NZVI/L in deionized water) was added to one serum bottle, resulting in an iron concentration (1 g/L, Fe^0 ∼40%) equal to that of the bare NZVI treatment group. The other bottle (without coated-NZVI addition) served as a control. Both treatments were placed in an anaerobic chamber at 25 °C and samples (1.8 mL) were taken periodically. RNA protective reagent (3.6 mL) (Ambion, Austin, TX) was added simultaneously to avoid RNA degradation. All experiments were repeated for verification purposes.

RNA Extraction, DNA Removal, and cDNA Synthesis. Total RNA was isolated from the 1.8-mL samples using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). To prevent adsorption of RNA onto NZVI (SI Figure S1), the NZVI particles (which are magnetic (33)) were removed prior to cell lysis using an Alnico Horseshoe Magnet (Fisher Scientific) placed at the bottom of the sample tube. The supernatant was transferred into another tube, and cells were then collected by centrifugation (Beckman Coulter Inc., Fullerton, CA) (5000g at 4 °C for 10 min). RNA isolation was carried out according to the manufacturer’s instructions. RNA samples were suspended in 50 µL of RNase-free water (Qiagen, Valencia, CA). The final DNA removal and First Strand cDNA synthesis were performed using a First Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario) according to the manufacturer’s instructions. Purified RNA and synthesized cDNA were stored at −80 °C prior to analysis by reverse transcriptase, quantitative PCR (RT-qPCR).

RT-qPCR. RT-qPCR was used to quantify the cDNA copy number of tceA, vcrA, and 16S rRNA gene in Dehalococcoides spp., using previously reported primer sequences (17, 34). RT-qPCR was performed on an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). Each reaction volume contained 10 µL of TaqMan Universal PCR Master Mix (Applied Biosystems); 0.7 µM (tceA, vcrA, or 16S rRNA) probe. Thermocycling conditions were as follows: 2 min at 50 °C, 15 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C (31).

Gene Expression Data Analysis. mRNA losses during sample preparation and inefficiencies of reverse transcription

![FIGURE 1. Relative tceA and vcrA expression fold changes after exposure to (a) bare NZVI; and (b) coated NZVI (1 g NZVI/L). Fold changes of target genes for each time point were normalized to initial conditions (time 0). All data points represent average values from triplicate samples, and error bars represent one standard deviation.](image)
often limit the accuracy of RT-qPCR (35–37). A useful method to discern the mRNA losses is adding luciferase control RNA as internal standard (38). However, the luciferase RNA was not an appropriate internal standard because of significant adsorption by NZVI (SI Figure S2). As an alternative, target mRNA quantities can be normalized to the quantity of mRNA of an endogenous housekeeping gene (36, 37). The ratio between target and housekeeping gene remains stable even when some mRNA are lost, so the relative quantification of target over housekeeping gene expression can overcome mRNA losses during sample preparation, including enzymatic or abiotic degradation and inefficiencies in reverse transcription.

The 16S rRNA gene of Dehalococcoides spp. was selected as an endogenous housekeeping gene because its transcript occurs at much higher level than RDase mRNA, regardless of growth stage and substrate availability (39). 16S rRNA gene expression was tested in the presence of bare NZVI, coated-NZVI, and coatings separately. The 16S rRNA remained stable over 168 h (p > 0.05, determined by one way ANOVA, SI Figure S3), demonstrating its suitability as a housekeeping gene. The amplification efficiency of target (\(tceA\) and \(vcrA\)) and housekeeping (16S rRNA) genes was statistically undistinguishable in the present of TCE (20 mg/L, dissolved in methanol) (SI Figure S4). Thus, \(tceA\) gene and \(vcrA\) mRNA was normalized to 16S rRNA gene within the same RNA extract.

Relative gene expression levels were calculated using the comparative \(C_T\) method (2\(^{-\Delta\Delta C_T}\) method (40), where \(\Delta\Delta C_T = (C_T - target - C_T - 16S)_{treatment} - (C_T - target - C_T - 16S)_{control}\). The expression of target genes in bare and coated NZVI treatments was normalized to that in the unexposed control group (SI Formulas S1 and S2). As a positive control for the relative quantification and RT-qPCR method, \(tceA\) and \(vcrA\) expression in the presence of TCE (20 mg/L, dissolved in methanol) was quantified (SI Figure S5). Both \(tceA\) and \(vcrA\) were significantly up-regulated by TCE exposure (\(p < 0.05\)), corroborating previous results obtained by absolute quantification and RT-qPCR (38, 41).

Whether up-regulation or down-regulation of gene expression was statistically significant relative to baseline or control conditions was determined using Student’s \(t\) test at the 95% confidence level.

**Results and Discussion**

### tceA and vcrA Expression after Exposure to NZVI

Magnetic separation of iron prior to Rt-qPCR precluded mRNA adsorption onto NZVI and interference on qPCR, which enabled quantification of gene expression. Thus, this novel pretreatment method could also benefit the study of other microbial—iron interactions relevant to corrosion and iron mineral cycling.

Significant down-regulation of \(tceA\) (\(p < 0.05\)) was observed following exposure to NZVI in the presence of residual chlorinated ethenes (Figure 1a), despite the biostimulatory effect of the cathodic \(H_2\) (405 ± 10 µmol, SI Figure S6) generated by NZVI corrosion (42). After 24 h, \(tceA\) expression relative to the housekeeping (16S rRNA) gene decreased by 1.6-fold compared to time 0, and reached the lowest level after 72 h with a dramatic 97-fold down-regulation. A higher degree of down-regulation was observed for \(vcrA\) (Figure 1a), with a 7.5-fold decrease in relative expression in 24 h and maximum 137-fold down-regulation after 72 h. These results are consistent with a previous study showing that TCE dechlorination by this mixed culture was inhibited by NZVI (1 g/L), with the overall first-order degradation rate coefficient decreasing by 54% from 0.115 ± 0.005 to 0.053 ± 0.003 h\(^{-1}\) (43).

### tceA and vcrA Expression after Exposure to Coated-NZVI

NZVI is commonly modified by surface coatings to prevent aggregation and enhance its distribution in contaminated aquifers (29, 43–46). Therefore, most NZVI that is used for aquifer remediation has engineered polymeric coatings. These coatings can significantly change the mobility and reactivity of NZVI in the subsurface, so understanding the toxicity of coated NZVI is essential to determining its environmental fate and impact.
Unlike the initial response to bare NZVI, both tceA and vcrA were significantly up-regulated in treatments with coated NZVI ($p < 0.05$); tceA expression increased 3-fold while vcrA increased 3.5-fold in 48 h compared to time 0 (Figure 1b). This up-regulation was likely due to the presence of the residual chlorinated solvents ([24, 47, 48]; cis-DCE (0.12 ± 0.03 mg/L), t-DCE (0.69 ± 0.11 mg/L), and VC (0.54 ± 0.16 mg/L). Accordingly, when olefin maleic acid copolymer coatings of NZVI were added alone, both tceA and vcrA were similarly up-regulated in the presence of these residual chlorinated solvents (Figure 2). Specifically, tceA expression increased 2.9-fold while vcrA increased 2.7-fold in 72 h, which is similar to the up-regulation associated with coated-NZVI exposure. Thus, the coating had no inhibitory or stimulatory effects on the RD2 gene expression.

Coated-NZVI had a slight inhibitory effect after 48 h ($p < 0.05$). The expression of tceA was down-regulated 10.6-fold, while vcrA was down-regulated 6.9-fold at 160 h compared to time 0. However, these levels of down-regulation were relatively small compared to those observed with bare NZVI (96-fold and 137.1-fold for tceA and vcrA, respectively). Thus, coating of NZVI with olefin maleic acid copolymer reduced the inhibition of tceA and vcrA gene expression. Coatings on NZVI electrostatically prevent direct contact between NZVI and cells ([11]), thus mitigating toxicity ([9, 10]). The observation of slight down regulation of tceA and vcrA in the presence of coated NZVI may be due to the eventual adhesion of a small number of NZVI to cells over the course of the experiment because coatings do not cover 100% of the NZVI surface.

The mechanism of tceA and vcrA gene expression inhibition by NZVI is unclear. Expressions may be lowered from decreased residual chlorinated ethene concentrations ([24, 47, 48]; cis-DCE: from 0.12 to 0.01 mg/L; t-DCE: from 0.69 to 0.51 mg/L; and VC: from 0.54 to 0.16 mg/L) or strong reducing conditions created at the membrane by direct interactions with NZVI that interfere with expression patterns of Dehalococcoides spp. More than 90 reductive dehalogenase-homologous genes have been identified in Dehalococcoides spp. genus ([22, 49, 50]) and most of the RDases are membrane-bound ([22, 48, 51]) and potentially susceptible to direct contact with bare NZVI. Previous studies with Dehalococcoides show that strong reductants (e.g., S$^2-$) greatly reduce halorespiration activity ([52]).

Cultures exposed to coated or uncoated NZVI were examined using TEM to visualize the association between the cells and nanoparticles. Because test conditions necessitate mixed cultures containing Dehalococcoides spp., it is difficult to distinguish the cells and clearly show association between NZVI and Dehalococcoides spp. To illustrate the potential for cell association of NZVI with gram negative bacteria such as Dehalococcoides (which is extremely difficult to isolate and is not commercially available in pure culture), a pure culture of E. coli was examined. E. coli is a well characterized bacterium that has been previously used as a model to study microbial–NZVI interactions ([9–11]). Micrographs show that exposure of E. coli to bare NZVI for 1 h resulted in attachment of nanoparticles to cells (Figure 3a), which is conducive to toxicity ([11]), whereas coated NZVI did not (Figure 3b). Moreover, some E. coli cells exposed to coated-NZVI are shown reproducing by binary fission, indicating that some cells continued to grow after exposure to coated-NZVI. These observations were representative of all observed fields on the copper grid. Overall, these results indicate that appropriate NZVI coatings may enable the concurrent or sequential participation of Dehalococcoides spp. in the cleanup process. Furthermore, this study shows a potential for coatings to reduce or eliminate the inhibitory effect of NZVI on biological dechlorination activity, which would consequently make coated-NZVI more favorable in remediation of chlorinated ethene contaminated sites.

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**Supporting Information Available**

Data for RNA adsorption onto NZVI, 16S rRNA, tceA, and vcrA expression after exposure to TCE, amplification efficiency of target and housekeeping genes, and H$_2$ production by NZVI (as well as details on gene expression analysis methodology). This material is available free of charge via the Internet at http://pubs.acs.org.

**Literature Cited**


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