# Supporting Information for:

# Impact of ZVI and sulfidated ZVI on sulfate-reducing microbial communities and implications for groundwater remediation

Nofil Khan[1]*, Asef Redwan[1,2]*, Syful Islam[3], Lingfei Fan[4], Weile Yan[4], and Kayleigh Millerick[1]**

* *Authors contributed equally to the paper*

[1]Department of Civil, Environmental and Construction Engineering, Texas Tech University, Texas, United States

[2]North South University, Dhaka, Bangladesh

[3]US Army Corps of Engineers, Buffalo, New York, United States

[4]Civil and Environmental Engineering, University of Massachusetts Lowell, Massachusetts, United States

** Corresponding Author. Tel: (806) 834-0370; Email: kayleigh.millerick@ttu.edu

## Preparation of ZVI

Prior to experimentation, $ZVI^{PLS}$ and $ZVI^{AA}$ were washed with 0.1% HCl (v/v) and shaken on a wrist action shaker for 20 min at 200 rpm (Islam et al., 2020). Acid washed particles were collected with magnets and the supernatant decanted. Particles were then washed with 10 mL deoxygenated DDI water (water purged with $N_2$ for 30 minutes prior to use) and re-collected for either sulfidation or direct use in experiments. nZVI particles were sonicated in methanol to break up loose aggregates and washed with deoxygenated DDI water to remove methanol, followed by particle collection and re-dispersion in an aqueous solution. nZVI that went through this washing procedure, but no other treatment is referred to as “as-synthesized” in this text.

## *Desulfovibrio* Maintenance

We maintained *D. desulfuricans* in Baar's Medium 2.0, which is a modified version of ATCC Medium: 1249, contains 1 g/L yeast extract, 3.50 g/L sodium lactate, 2.40 g/L HEPES free acid, 5.0 g/L sodium citrate dihydrate, 1.0 g/L calcium sulfate dihydrate, 1.0 g/L ammonium chloride and 0.2 g/L monopotassium phosphate. The final pH of the media was adjusted to 7.5, and media was dispersed into serum bottles, degassed with $N_2$ for 20 min., sealed anaerobically with butyl stoppers. and autoclaved (121 $^{o}$C, 20 min). *D. desulfuricans* was amended into media (2%, v/v) and incubated at 28 $^{o}$C. *D. desulfuricans* cells were grown to late log phase (approximately 120 hours) prior to transfer, and a standard growth curve for *D. desulfuricans* is provided below in **Figure SI-1**.

## Sulfate Measurements

To assess biological sulfate rates, 1 mL of media was periodically removed from reactor vials. Sample aliquots were centrifuged for 2 minutes at 5,000 rpm to precipitate iron particles and cells. The supernatant was collected and filtered using 0.2 µm PTFE syringe filters and diluted 1:10 with DDI water. Sulfate was analyzed via ion chromatography (AS40, Dionex, Sunnyvale, California) using an Dionex™ IonPac™ AS14A column (4 x 250 mm) and a carbonate/bicarbonate (8 mM/1 mM) isocratic mobile phase at 1 mL/min. Cell-free experiments using sulfidated and as-synthesized particles (5 g particles per L media) were prepared in duplicate to confirm that all sulfate transformation was biologically mediated.

To calculate first-order rate constants, we assumed an initial lag period and did not include data from t < 20 hours. Data fitted were from the time points collected following this lag, representing ~60 hours of activity, and this approach generated coefficients of determination ($R^2$ values) that typically exceeded 0.9. Data (six points per time course) were fit to the first order equation described below:

$$\mathbf{ln\ [C/C_o] = -\ \mathit{k}t;} \qquad \mathbf{(1)}$$

In this equation, t represents time, $k$ is the reaction rate coefficient ($hr^{-1}$ or $day^{-1}$), C is the sulfate concentration (mM) at time $t$, and $C_o$ is the initial concentration of sulfate (mM).

## Methodology for 16S rRNA Gene Survey Sequencing and Raw Sequence Data Processing

*DNA Extraction (AMR-1 baseline community and experiments with Peerless and n-ZVI).* DNA was extracted from 0.5 mL supernatant (experimental) and 0.5 mL deionized water (controls) using the DNeasy PowerSoil kit (Qiagen) following manufacturer's instructions. Extracted DNA was quantified using a Qubit™ 3.0 fluorometer (Life Technologies) to verify successful extraction. No DNA was detected in negative controls, and water samples were not submitted for sequencing. DNA from in-house extracts were stored at -20 °C prior to platform sequencing.

*Sequencing.* DNA extracts were paired-end sequenced using the MiSeq Double PCR method and the Illumina MiSeq Reagent kits (Illumina, San Diego, CA). RTL Genomics (full-service sequencing laboratory; Lubbock, TX) performed all sequencing. PCR was performed using primers that target the variable V3-V4 regions of the 16S bacterial rRNA gene. The AMR-1 baseline community and experiments using Peerless and n-ZVI were amplified with primers 357wF and 785R, which correspond to sequences 5'-CCTACGGGNGGCWGCAG-3' (forward) and 5'-GACTACHVGGGTATCTAATCC -3' (reverse), which are used for sediment incubations and target a region of 428 bp length.(Navarrete-Euan et al., 2021) Experiments with Alfa Aesar particles and one cell control were amplified with primers 515F and 806R, which correspond to sequences 5'-GTGCCAGCMGCCGCGGTAA-3' (forward) and 5'-GGACTACHVGGGTWTCTAAT-3' (reverse). Samples were run individually (no replicate analysis) and were not pooled, and PCR products were not quantified. The read length of the MiSeq platform was 2x300 bp.

*Raw Sequence Data Processing.* Quality control and filtering of raw sequencing data were performed using QIIME 2 (version 2022.02) to ensure the generation of high-quality data for downstream analyses (Bolyen et al., 2019). Raw sequencing data, in FASTQ format, were imported into the QIIME 2 environment using the QIIME 2 Artifact API or the graphical user interface. Data trimming was performed using the DADA2

plugin in QIIME 2 (Callahan et al., 2016). The DADA2 algorithm was chosen for its ability to accurately correct errors, denoise data, and remove low-quality reads. Prior to determining the trimming parameters, the quality scores were visualized using QIIME 2's quality control tools, specifically the q2-demux plugin. The parameters <trim_left_f>, <trim_left_r>, <trunc_len_f>, and <trunc_len_r> were adjusted based on the quality profiles of the sequencing data. The command generated three output files: table.qza (feature table), rep-seqs.qza (representative sequences), and denoising-stats.qza (summary of denoising statistics). To ensure equal sequencing depth across samples, rarefaction was performed on the processed data. The "table.qza" file generated in the previous step was rarefied using the QIIME 2 plugin of choice (e.g., q2-diversity) (Caporaso et al., 2010). The rarefaction depth was set to the lowest sequencing depth among all samples to maintain data integrity and avoid biased results. The trimmed and rarefied data were then subjected to various visualization and analysis tools available in QIIME 2. Alpha and beta diversity analyses were conducted to assess within-sample diversity and between-sample dissimilarity, respectively. Taxonomic classification was performed on previously generated "rep-seqs.qza" with the Naive Bayes classifier in QIIME 2, to assign taxonomy to the representative sequences using 16S reference database from SILVA (https://www.arb-silva.de/). Following bioinformatics, one-way ANOVA analyses were performed on triplicate operational taxonomic unit (OTU) recoveries using Tukey's multiple comparisons test and GraphPad Prism 8.0.1 software.

## Methodology for XPS Analysis

Iron materials were dried after synthesis or sulfur treatment in a glovebox purged with high purity $N_2$ for up to 48 h. The dried solids were stored in $N_2$-fillled gastight vials prior to analysis. Sample preparation followed the procedure we described previously (Han and Yan, 2016, 2014). $Fe2p_{3/2}$ and S2p region spectra were acquired at a 45° takeoff angle and 20 eV pass energy. The software package CasaXPS (Version 2.3.25PR1.0) was used for XPS spectral analysis. The binding energy was referenced to the C1s peak of adventitious carbon at 284.8 eV. The S2p doublets were fitted using an energy split of 1.2 eV and an area ratio of 2:1 (Han and Yan, 2016).

## Methodology for SEM Microscopy

To prepare, samples were washed with 0.05 M cacodylate buffer three times, then fixed by 2.5% glutaraldehyde solution in 0.05 M cacodylate buffer for 24 h. The fixed samples were further washed with 0.05M cacodylate buffer three times to remove excess glutaraldehyde, followed by post fixation with 1% osmium tetroxide ($OsO_4$) in 0.05 cacodylate buffer for 30 min. After washing with water three times to remove excess $OsO_4$, the sample solutions were then frozen into solid ice by liquid nitrogen and then died overnight by freeze dryer. The fixed and dried sample powders were placed on carbon tape, mounted onto an aluminum stud, and coated with a thin layer of Iridium (Ir) prior to imaging under a Hitachi S-4300 SE/N high resolution field emission SEM (Hitachi High-Tech in America, Dallas, TX). Quartz PCI Digital Image Capture (Quartz Imaging Corporation, Vancouver, Canada) was used for data collection.

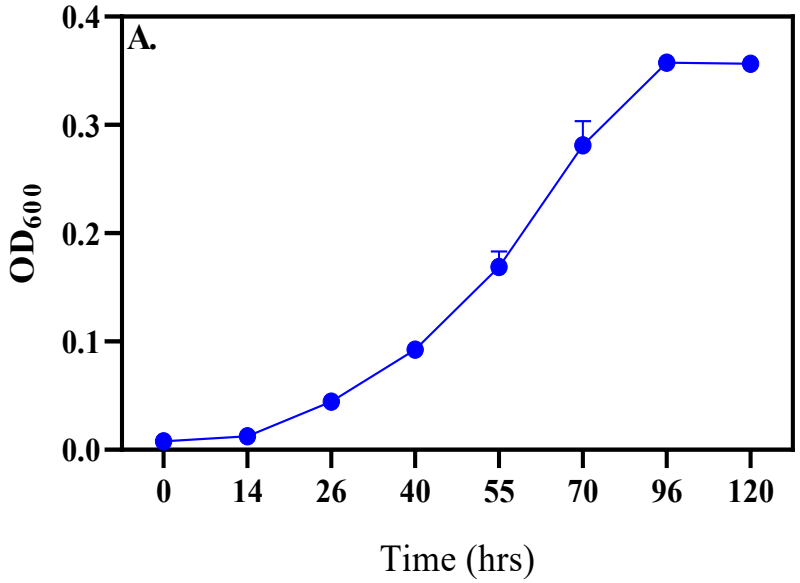



**Figure SI-1.** Typical growth curve for *Desulfovibrio desulfuricans* obtained from $OD_{600}$ readings.

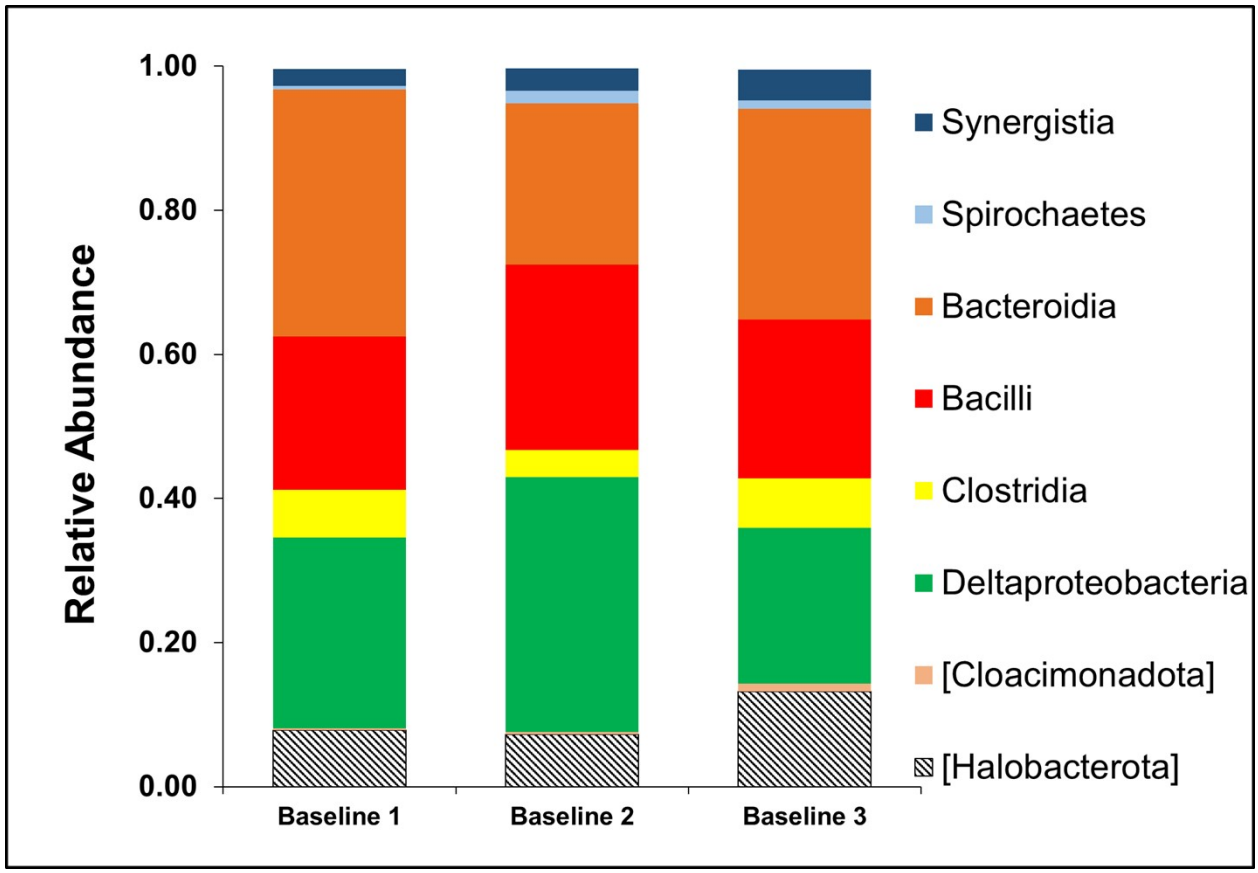



**Figure SI-2.** Baseline community composition of AMR-1 prior to ZVI amendment (Islam et al., 2021).

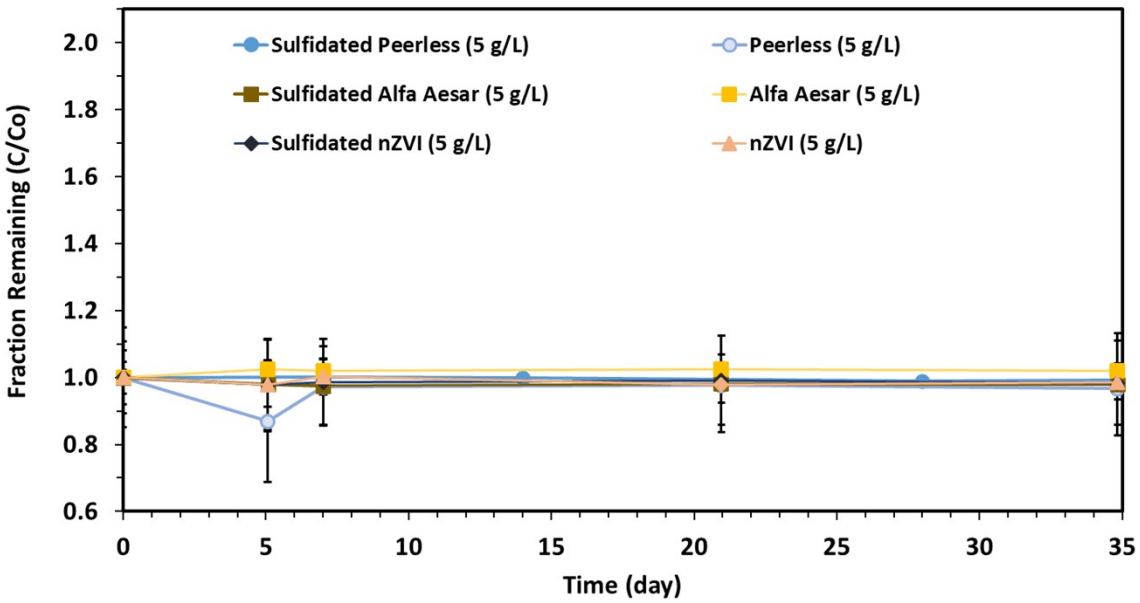
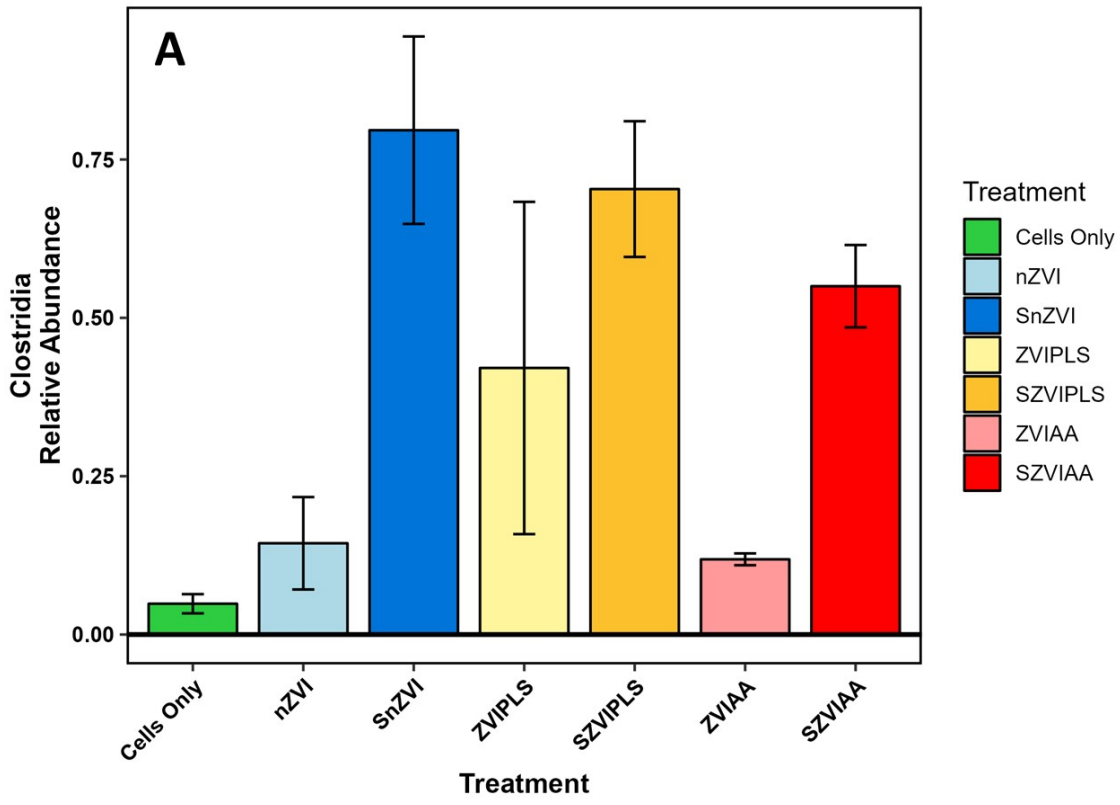
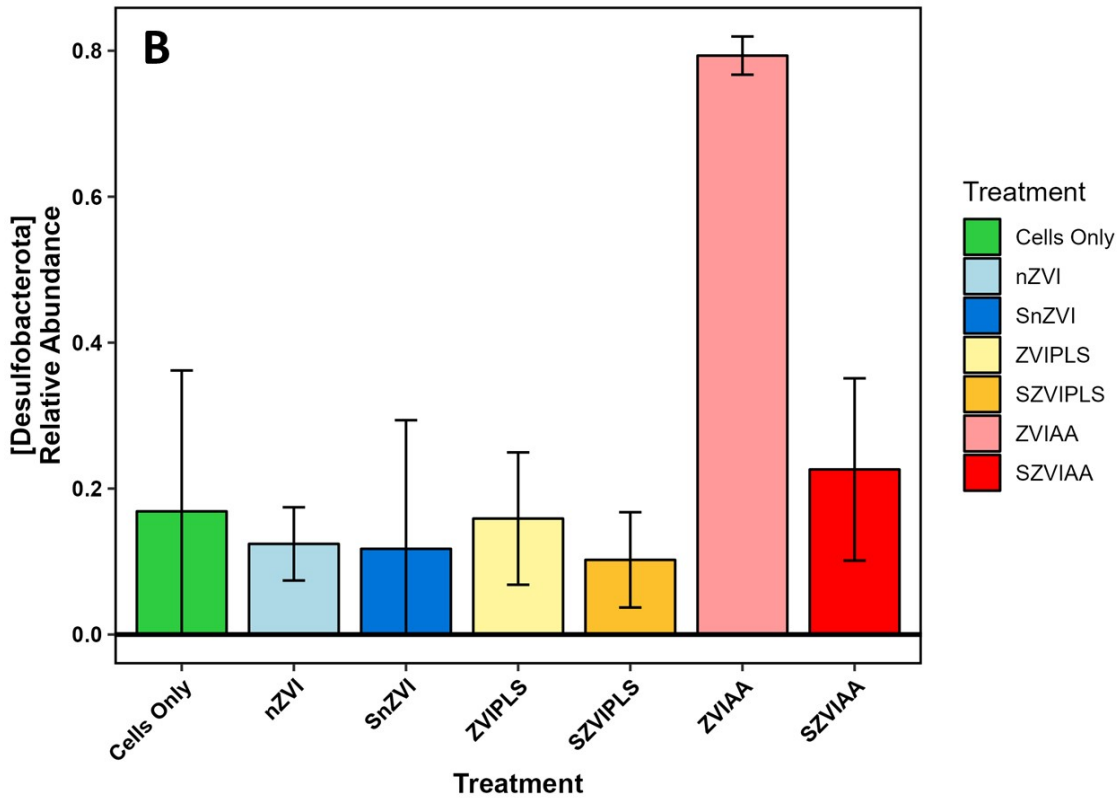



**Figure SI-3.** Time course showing sulfate concentrations in media without cells in the presence of $ZVI^{PLS}$, $ZVI^{AA}$, and nZVI.


A
Clostridia
Relative Abundance
0.75
0.50
0.25
0.00
Cells Only
nZVI
SnZVI
ZVIPLS
SZVIPLS
ZVIAA
SZVIAA
Treatment
Treatment
Cells Only
nZVI
SnZVI
ZVIPLS
SZVIPLS
ZVIAA
SZVIAA


B
[Desulfobacterota]
Relative Abundance
0.8
0.6
0.4
0.2
0.0
Cells Only
nZVI
SnZVI
ZVIPLS
SZVIPLS
ZVIAA
SZVIAA
Treatment
Treatment
Cells Only
nZVI
SnZVI
ZVIPLS
SZVIPLS
ZVIAA
SZVIAA

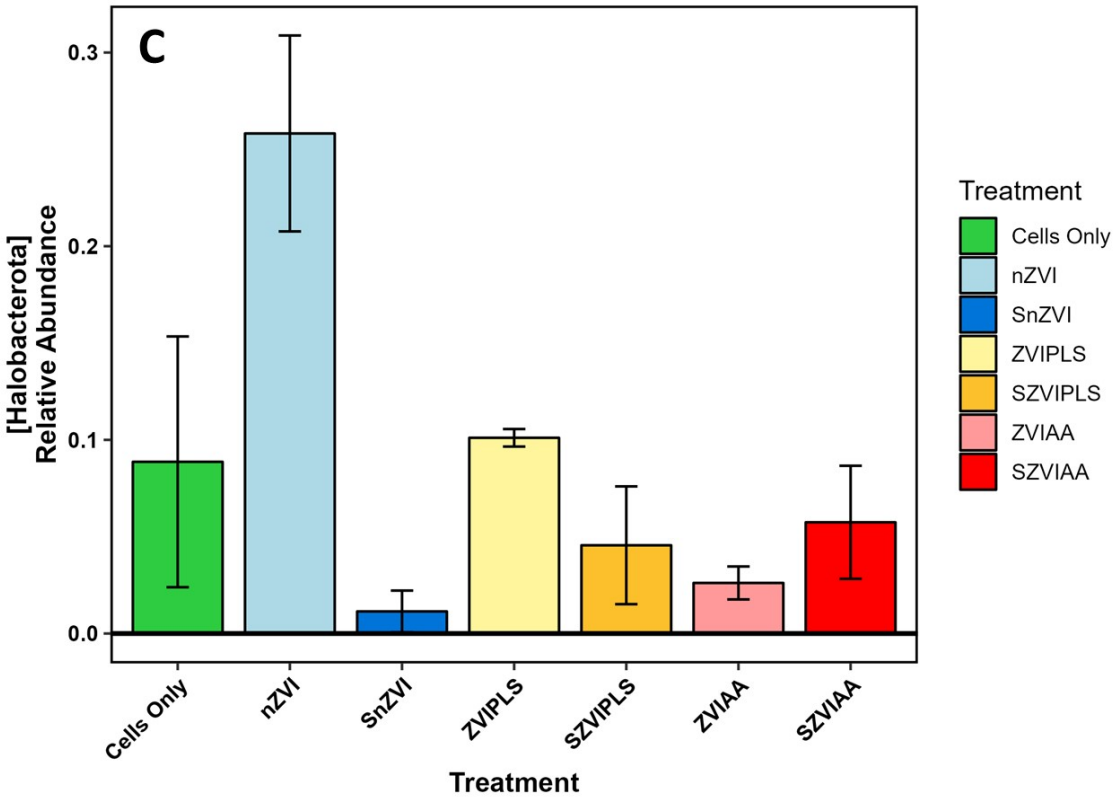



**Figure SI-4.** Relative abundance of key phyla across all treatments after 28 days of inc. Panels include phylum Clostridia (Panel A), Desulfobacterota (Panel B), and Halobacterota (Panel C).

**Table S1-1**. Physiochemical properties of ZVI particles (Islam et al., 2020; Li et al., 2009)

| ZVI Type | Average Diameter (μm) | BET Surface Area[a] ($m^2$ $g^{-1}$) | Fe Content (wt. %) | Metal Impurities[c] (wt. %) | | |
|---|---|---|---|---|---|---|
| | | | | Mn | Ni | Cu |
| Nanoscale ZVI (nZVI) | 0.055 ± 0.045 | 25-35 | ~99.5 | BDL | BDL | BDL |
| Alfa Aesar ($ZVI^{AA}$) | 3.80 ± 1.07 | 0.347 ± 0.001 | ~99.5[b] | BDL | BDL | BDL |
| Peerless 50D ($ZVI^{PLS}$) | 3.25 ± 2.5 | 1.95 ± 0.003 | >90[b] | 0.67 ± 0.06 | 0.19 ± 0.02 | 0.15 ± 0.02 |

[a] Estimated based on 11-point $N_2$ adsorption curves.
[b] Based on specifications provided by the manufacturer.
[c] Measured via acid digestion and ICP-MS analyses. BDL stands for below detection limit, which is 0.003%, 0.0002%, and 0.011% for Mn, Ni, and Cu, respectively.
[d] Determined as the surface-area weighted mean diameter using an aerosizer.

**Table SI-2.** OTU recovery and ANOVA comparison of OTU recovery (Tukey's multiple comparisons test) from enrichment culture AMR-1 following 28 days of exposure to iron particle treatments.

*Composition (total OTUs and OTUs classified by domain) of communities following exposure to ZVI particles*

| Treatments | Total OTUs | Eubacterial OTUs | Archaeal OTUs | Archaea (%) |
|---|---|---|---|---|
| No Amendment | 62,686 ± 5,531 | 56,876 ± 2,597 | 5,809 ± 3,929 | 8.7 ± 5.2 |
| nZVI | 56,804 ± 6,424 | 41,891 ± 2,568 | 14,912 ± 4,105 | 25.8 ± 4.1 |
| Sulfidated nZVI | 57,728 ± 8,591 | 57,061 ± 8,492 | 667 ± 486 | 1.1 ± 0.9 |
| $ZVI^{PLS}$ | 55,128 ± 5,306 | 49,570 ± 4,917 | 5,558 ± 414 | 10.1 ± 0.4 |
| Sulfidated $ZVI^{PLS}$ | 45,972 ± 5,158 | 43,810 ± 4,481 | 2,162 ± 1,328 | 4.6 ± 2.5 |
| $ZVI^{AA}$ | 65,145 ± 12,837 | 63,921 ± 12,250 | 1,224 ± 880 | 1.7 ± 1.3 |
| Sulfidated $ZVI^{AA}$ | 58,416 ± 17,801 | 55,468 ± 17,827 | 2,948 ± 320 | 5.7 ± 2.4 |

*One-way ANOVA of total OTUs and OTUs classified by domain between treatments (p-values shown below)*

| Treatments | Total | Eubacterial | Archaeal |
|---|---|---|---|
| No Amendment vs. $ZVI^{PLS}$ | .784 | .664 | >.999 |
| No Amendment vs. nZVI | .936 | .122 | **.049** |
| $ZVI^{PLS}$ vs. nZVI | .995 | .625 | **.043** |
| nZVI vs. Sulfidated nZVI | >.999 | .117 | **.004** |
| $ZVI^{PLS}$ vs. Sulfidated $ZVI^{PLS}$ | .681 | .814 | .701 |
| $ZVI^{AA}$ vs. Sulfidated $ZVI^{AA}$ | .937 | .706 | .985 |

Numbers in **bold** indicate OTU recoveries are statistically significant (95% confidence interval).

## References


Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K.B., Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.-X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., Van Der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., Von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37, 852–857. https://doi.org/10.1038/s41587-019-0209-9

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336. https://doi.org/10.1038/nmeth.f.303

Han, Y., Yan, W., 2016. Reductive Dechlorination of Trichloroethene by Zero-valent Iron Nanoparticles: Reactivity Enhancement through Sulfidation Treatment. Environ. Sci. Technol. 50, 12992–13001. https://doi.org/10.1021/acs.est.6b03997

Han, Y., Yan, W., 2014. Bimetallic nickel–iron nanoparticles for groundwater decontamination: Effect of groundwater constituents on surface deactivation. Water Res. 66, 149–159. https://doi.org/10.1016/j.watres.2014.08.001

Islam, S., Han, Y., Yan, W., 2020. Reactions of chlorinated ethenes with surface-sulfidated iron materials: reactivity enhancement and inhibition effects. Environ. Sci. Process. Impacts 22, 759–770. https://doi.org/10.1039/C9EM00593E

Islam, S., Redwan, A., Millerick, K., Filip, J., Fan, L., Yan, W., 2021. Effect of Copresence of Zerovalent Iron and Sulfate Reducing Bacteria on Reductive Dechlorination of Trichloroethylene. Environ. Sci. Technol. https://doi.org/10.1021/acs.est.0c07702

Li, S., Yan, W., Zhang, W., 2009. Solvent-free production of nanoscale zero-valent iron (nZVI) with precision milling. Green Chem. 11, 1618–1626. https://doi.org/10.1039/B913056J

Navarrete-Euan, H., Rodríguez-Escamilla, Z., Pérez-Rueda, E., Escalante-Herrera, K., Martínez-Núñez, M.A., 2021. Comparing Sediment Microbiomes in Contaminated and Pristine Wetlands along the Coast of Yucatan. Microorganisms 9, 877. https://doi.org/10.3390/microorganisms9040877