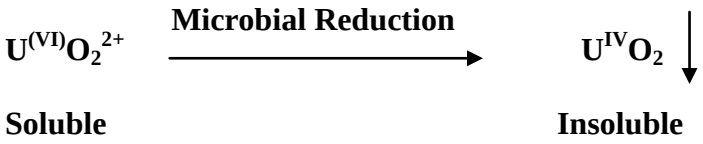
PROBLEM AND RESEARCH OBJECTIVES

Uranium is an important environmental contaminant impacting groundwater supplies in Arizona. The main sources are from uranium mine tailings, former uranium processing plants, and high natural background levels in areas of granite bedrock [1]. In the environment, uranium generally occurs as hexavalent uranium (U(VI)) or tetravalent uranium (U(IV), often present as the mineral uraninite, UO_2). While U(VI) is soluble and mobile, U(IV) is highly insoluble and immobile [2]. Therefore, reductive precipitation is an attractive approach to remove soluble uranium and remediate contaminated groundwater [3]. Reduction of soluble U(VI) can be catalyzed by chemical and by microbial processes involving anaerobic bacteria [4-6]. Typically organic substrates (e.g. ethanol, lactate, acetate) are utilized as the electron donors to drive biological uranium reduction [7].



Several studies have proven that zero-valent iron (ZVI, Fe^0) is an effective reactive material for the immobilization of U(VI) [8]. ZVI is most commonly applied in permeable reactive barriers [9]. The removal of U(VI) by these methods has been found to be mainly due to reductive precipitation [10-11], although co-precipitation with iron corrosion products has been also found to be an important mechanism in some of the studies [12]. However, the kinetic limitations of the chemical reductive precipitation are a constraint to these methods [13]. While ZVI is a well-known source for electron equivalents for many microorganisms in the environment [14-15], its use by uranium-reducing microorganisms has not been defined.

Preliminary work by our research group has led to the enrichment of a novel uranium-reducing bacterial culture that is capable of utilizing ZVI as an electron donor [16]. The microbial culture greatly accelerates uranium reduction rates with ZVI by more than 20-fold in a sustained fashion. ZVI has some important advantages over alternative bioremediation strategies relying on organic electron donors. The ZVI could provide a

long-term reservoir of slow-release electron equivalents as well as buffer against uranium re-oxidation.

OBJECTIVES

The objective of this study is to investigate the use of nano-sized ZVI (nZVI) as an electron donor for uranium-reducing microorganisms. Stabilized dispersions of nZVI can be transported through porous media to facilitate in situ bioremediation of uranium-contaminated groundwater. This project is expected to lead to the development of a low-cost and low-maintenance method for the in situ bioremediation of groundwater contaminated by uranium, which generates insoluble uranium minerals that are stable against re-oxidation over prolonged time periods. Application of this technique could be expanded to the treatment of other toxic contaminants amenable to microbial reductive processes (e.g., perchlorate, arsenate, oxidized radionuclides).

METHODOLOGY

Source of inoculum. Inoculum for the experiments were obtained from enrichment cultures developed from the effluent of a continuous ZVI/sand packed column that reduced U(VI) with ZVI as electron donor [16]. A 16S-rRNA bacterial clone library performed prior on this enrichment culture proved that it was composed of two major bacterial genera (*Dechloromonas* and *Stenotrophomonas*, publication in progress); due to this reason, the inoculum is referred to as a bacterial co-culture.

Basal media. The mineral media used in the batch experiments was adapted from previous works [16]. The composition of the media was the following (in mg L^{-1}): NH_4HCO_3 (5.0), K_2HPO_4 (2.0), $Ca(OH)_2$ (1.0), yeast extract (1.67) and $MgCl_2 \cdot 7H_2O$ (41.0). The concentration of the trace element solution was (in mg L^{-1}): H_3BO_3 (0.01), $FeSO_4 \cdot 7H_2O$ (0.56), $Na_2WO_4 \cdot H_2O$ (0.10), $ZnSO_4 \cdot 7H_2O$ (0.02), $MnSO_4 \cdot 7H_2O$ (0.08), EDTA (0.20), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.04), $AlK(SO_4)_2 \cdot 12H_2O$ (0.04), $NiSO_4 \cdot 6H_2O$ (0.02), $CoSO_4 \cdot 7H_2O$ (0.47), $Na_2SeO_3 \cdot 5H_2O$ (0.02), $CuSO_4 \cdot 5H_2O$ (0.03), and resazurin (0.04). After adjusting to a pH value of 7.5, the media was sterilized in an autoclave (Yamato Scientific America Inc., Santa Clara, CA) at 120°C for 20 min. After cooling

down, it was amended with a filter-sterilized $NaHCO_3$ solution to a final concentration of 1.0 g L^{-1}.

Batch experiments. All batch microcosms were performed in 160-mL sterilized serum bottles (Wheaton, Millville, NJ), consisting in 100 mL of basal media and 60 mL of headspace. These consisted in treatments with 1 mM of ZVI (either micron-sized or nano-sized ZVI, depending on the conditions tested) and aliquots from a 10 mM stock solution to get final concentration of 30.0 μM U(VI). Anaerobic conditioning of the headspace was carried out by flushing a N_2/CO_2 gas mixture as described previously [6]. For biological treatments or controls, aliquots corresponding to 5% v/v of planktonic inoculum from an active treatment were added to each bottle inside an anaerobic glove box (COY Laboratory Products Inc., Grass Lake, MI) after the anaerobic flushing. Finally, the headspace was re-conditioned with N_2/CO_2 gas mixture at the end of the inoculum addition to replenish anoxic conditions. Controls consisting in non-inoculated, ZVI-only, as well as inoculum only (endogenous control) were set-up for each transfer. All treatments and controls were carried out in duplicated replicates, and incubated statically in the dark at 30°C. Soluble U was measured over time during the whole experiment. For biological experiments with polyethylenimine (PEI) as dispersant for nZVI, aliquots from a 400 mg L^{-1} PEI stock solution were added to the media prior to sterilization for a final concentration of 11.2 mg L^{-1} (ratio nZVI/PEI of 5:1).

Soluble U(VI) analysis. Liquid samples were taken into Eppendorf ™ centrifuge tubes, being then centrifuged at 10,000 rpm (RCF of 10,621 x g) for 10 min. After transferring the supernatant to a 3% HNO_3 solution, soluble U was measured by using an Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES) system model Optima 2100 DV from Perkin-Elmer™ (Shelton, CT, USA) at a wavelength of 385.958 nm. This technique is based on the electromagnetic radiation emission or absorption by an ion in solution. Since U(VI) is being consumed through redox transformation to an insoluble specie U(IV), the reduction process was monitored by measuring the intensity of the remaining soluble uranium. The detection limit for U was 10 μg L^{-1}.

Dispersions preparation. For the particle size distribution (PSD) determinations, nanoparticle dispersions of nZVI were prepared both in MilliQ water and in the biological media used in the bioassays. Polyethylenimine (PEI) and polyacrylic acid

(PAA) were tested as dispersants for the nanoparticles. The following dispersions were prepared: nZVI with MilliQ water, nZVI with water and PEI, nZVI with water and PAA, nZVI with medium, nZVI with medium and PEI, and nZVI with medium and PAA. For this purpose, a 1000 mg L^{-1} nZVI stock solution was prepared at pH 7.2, and then the solution was sonicated for 5 min at 70% of amplitude in an ultrasonic processor. Also, 400 mg L^{-1} PEI and PAA stock solution was used. The final concentrations of nanoparticles and dispersant were 100 and 20 mg L^{-1}, respectively.

PSD determination. The particle size distribution of the nanoparticle dispersions was measured by dynamic light scattering (DLS) using Zeta Sizer Nano ZS instrument (Malvern, Inc., Sirouthborough, MA). PSD measurements were performed using the same instrument. DLS analyzes the velocity distribution of particle movement by measuring dynamic fluctuations of light scattering intensity caused by the Brownian motion of the particle. The technique yields a hydrodynamic diameter that is calculated via the Stokes-Einstein equation from the aforementioned measurements. The unit employs a 4mW He-Ne laser with a wavelength of 633 nm, and a measurement angle of 173°.

pH. Measurements were performed in a VWR SympHony SB20 electrode as indicated by Standard Methods [17].

Chemicals. Uranium (VI) was purchased in form of uranyl chloride trihydrate ($UO_2Cl_2 \cdot 3H_2O$) from International Bio-Analytical Industries Inc. (Boca Raton, FL). Nano-sized ZVI (Fe^0, 40-60 nm, 99.9% purity) was purchased from SkySpring Inc (Houston, TX, USA). Polyethylenimine (PEI, branched, $H(NHCH_2CH_2)_nNH_2$) and ZVI powder (Fe^0, <10 μm, 99.9+% purity) were purchased from Sigma Aldrich Co. (St. Louis, MO). Poly(acrylic) acid powder (MW ~1800) was purchased from Sigma Aldrich (St Louis, MO). Ammonium bicarbonate (NH_4HCO_3, 21.30-21.73% as NH_4^+), sodium hydroxide (NaOH), methylene blue ($C_{16}H_{18}ClN_3S \cdot 3H_2O$) and nitric acid ($HNO_3$, 70%) were supplied by Fisher Chemical (Fair Lawn, NJ). Magnesium sulfate ($MgSO_4 \cdot 7H_2O$), calcium hydroxide ($Ca(OH)_2$), potassium phosphate dibasic (K_2HPO_4, >99.0%) and potassium nitrate (KNO_3, 99.0%), were purchased from J.T. Baker (Phillipsburg, NJ). Yeast extract was supplied from BD (Sparks, MD). Sodium bicarbonate was obtained from Pfaltz & Bauer (Waterbury, CT).

PRINCIPLE FINDINGS AND SIGNIFICANCE

1. Evidence of biological enhancement of U(VI) reduction with ZVI

Preliminary experiments were carried out to evaluate the capacity of the microbial co-culture to accelerate the reduction of U(VI) with ZVI. Figure 1 shows an example of an assay carried out with micron-sized ZVI and U(VI). As can be observed from this plot, uranium is more rapidly reduced in the presence of the enrichment co-culture, compared to the abiotic incubation with ZVI. Neither biological controls without electron donor (endogenous) or abiotic controls without ZVI could remove any significant U(VI) during the experimental period. These experiments revealed that there is an enhanced rate of reduction by the incorporation of the enrichment culture over the abiotic rate of uranium reduction.

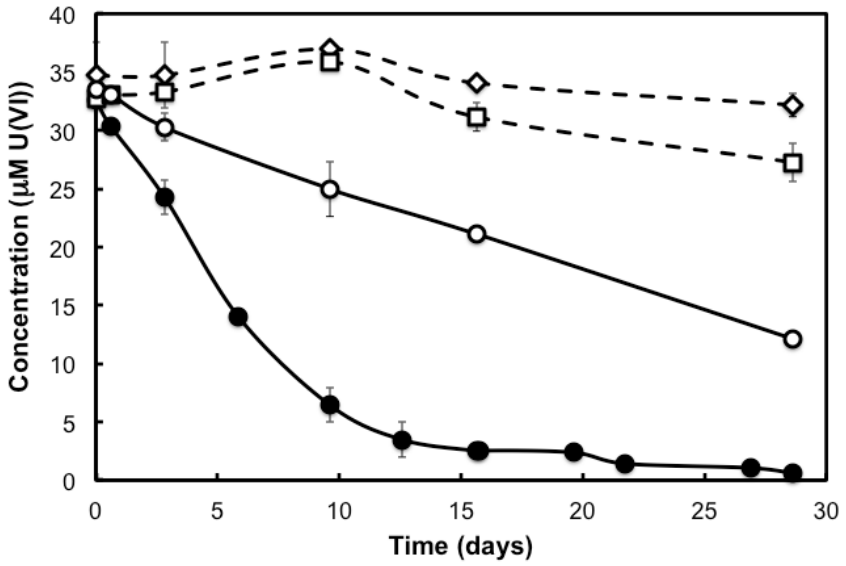


Fig. 1. Removal of soluble U(VI) by the microbial co-culture with ZVI.
Legends: ---◊---, Abiotic, no Fe^0; ---□---, Biological, no Fe^0; —○—, Abiotic with Fe^0; —●—, Biological with Fe^0.

2. Biological enhancement of U(VI) reduction with nano-ZVI

2.1. Comparison of different sources of ZVI

Two experiments were done comparing the effectiveness of micron-sized ZVI in reducing U(VI) versus that of nano-sized ZVI. In the first experiment, thin septa were used to seal the bottles from the atmosphere. The results of this experiment can be found in Figure 2. The thin septa that were used were not sufficient to seal off leaks of gas, and oxygen was able to seep into the bottles. Initially, it can be seen that the U(VI) was reduced in the bottles containing either size of ZVI and in those containing the iron with the enrichment co-culture. However, oxygen leaking into the bottles caused the U(IV) to reoxidize to U(VI), which accounts for the periodic increases in the U(VI) concentration.

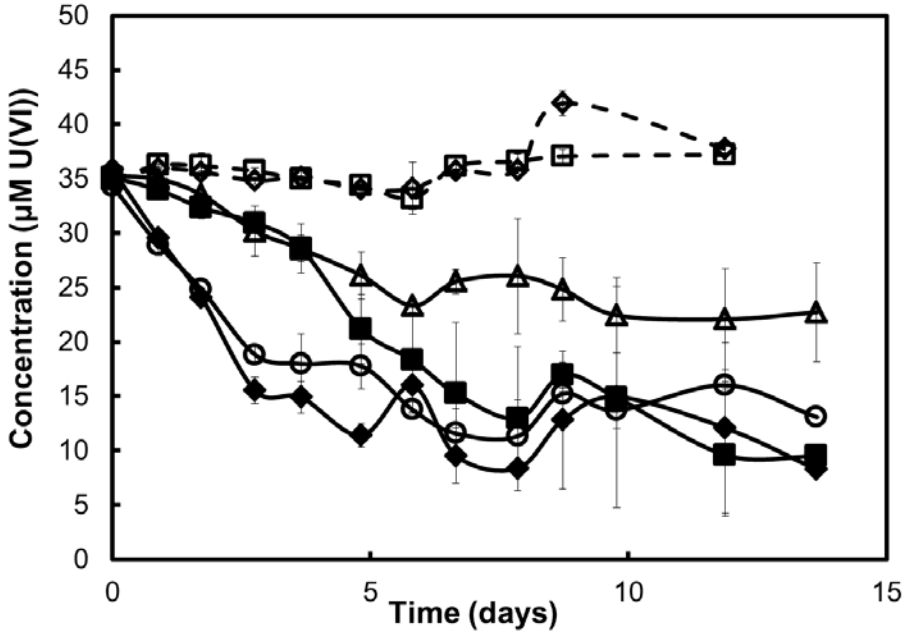


Fig. 2. Removal of soluble U(VI) by the microbial co-culture under uncontrolled atmospheric conditions with micron-ZVI versus nano-ZVI.
Legends: --◊--, Abiotic, no Fe^0; --□--, Biological, no Fe^0; —○—, Abiotic with nZVI; —Δ—, Abiotic + micron-Fe^0, —♦—, Biological + nZVI; —■—, Biological + micron-Fe^0.

In the second experiment, thicker septa were used in order to ensure that conditions within the bottles were kept anaerobic. The results of this experiment can be found in Figure 3. As can be seen in this plot, the removal of U(VI) occurred faster in the

bottles containing the enrichment culture and nZVI than in the abiotic bottles containing nZVI. The same effect can be seen when comparing the bottles containing the enrichment culture and micron-sized ZVI to the abiotic bottles containing micron-sized ZVI. This shows an enhancement in U(VI) reduction rate in the presence of co-culture with either type of ZVI. Additionally, there was faster reduction in the abiotic bottles containing nZVI than in the abiotic bottles containing micron-sized ZVI. Removal also occurred faster in those bottles containing the enrichment culture with the nZVI than in those containing the enrichment culture and micron-sized ZVI. However, this increase in uranium removal in the bottles with enrichment culture and nZVI is shown to happen only for the first seven days of the experiment before uranium levels in the bottles containing enrichment culture and micron-sized ZVI became the same as those observed with treatments with nZVI. After this, neither treatment appears to reduce faster than the other. Lastly, the lack of U(VI) removal in the endogenous controls and the abiotic controls without ZVI confirm the results of the inactivity controls as was found in the preliminary experiments.

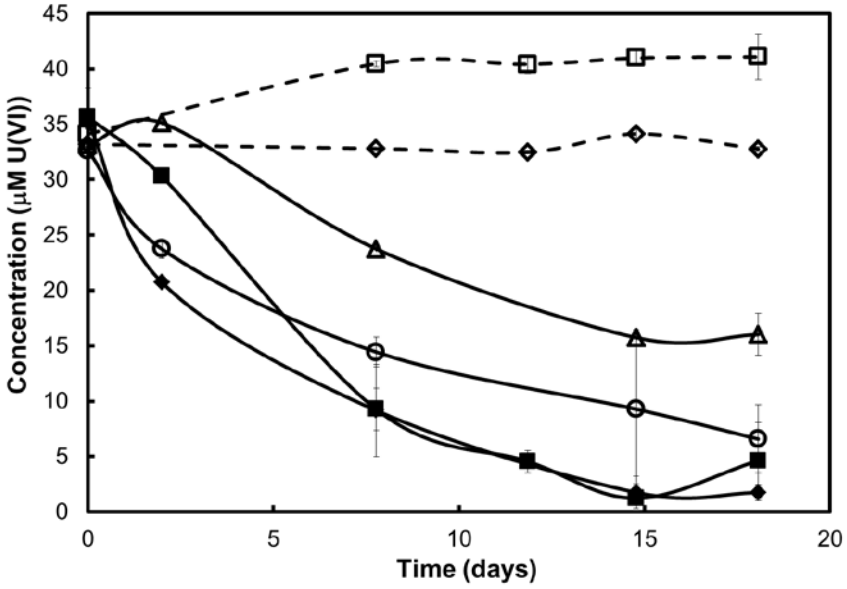


Fig. 3. Removal of soluble U(VI) by the microbial co-culture under anaerobic conditions with micron-ZVI versus n-ZVI.
Legends: ---◊---, Abiotic, no Fe^0; ---□---, Biological, no Fe^0; —○—, Abiotic with nZVI; —Δ—, Abiotic with micron-Fe^0; —♦—, Biological with nZVI; —■—, Biological with micron-Fe^0.

2.2. Characterization of nano-sized ZVI (PSD) and use of dispersants to enhance nZVI dispersion stability

A DLS test was carried out to determine the PSD of nZVI in MilliQ water and in the normal basal medium used in the bioassays. Table 1 summarizes the particle size values at the different conditions tested. The average PSD of nZVI provided by the manufacturer ranges from 40-60 nm. DLS measurements revealed that particles aggregate in water (pH 7.4) as well as in the medium (pH 8.7) used in bioassays. Polymeric surfactants may be used to modify the surfaces of the nanoparticules in order to counteract the aggregation tendency by the normal van der Waals forces.

In this way, two dispersants were used to improve the stability of nZVI: PEI (cationic) and PAA (anionic), either with water or the basal medium from bioassays. The plot displayed in Figure 4 shows the differences in PSD obtained in mineral medium, based on the mean intensity.

Table 1. Average particle size (hydrodynamic diameter) of nZVI in water and biological medium in the presence and absence of dispersant addition.

	Diameter (nm)	
Conditions tested	**Average**	**Std Dev**
Water	829	±130
Water-PEI	1295	±267
Water-PAA	1317	±267
Medium	1383	±144
Medium-PEI	1249	±302
Medium-PAA	2803	±1054

From results obtained in these tests with the basal medium, it could be observed that a marginally lower level of aggregation is possible with PEI. In this way, this cationic dispersant may increase the stability of the nZVI at the pH conditions of the biological assays for the treatment of U(VI).

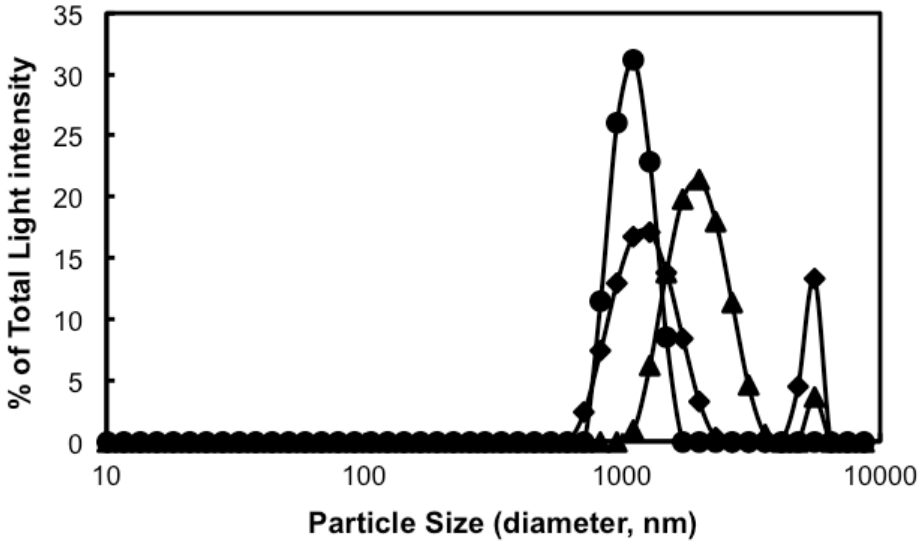


Fig. 4. Comparison of the PSD achieved with different dispersants in media (pH 8.7). Legends: —◆—, ZVI with media only; —●—, ZVI with PEI and media; —▲—, ZVI with PAA and media.

2.3. Use of PEI as dispersant for nZVI

An experiment was conducted to determine if the addition of PEI to the enrichment culture with nZVI enhances the removal capacity of this treatment. The results for those bottles that did not contain PEI can be found in Figure 5, while those for the bottles that did contain PEI are in Figure 6. For those bottles without PEI, there was no removal of U(VI) in the endogenous and abiotic bottles without nZVI. Also, there was an increase in removal in those bottles containing the co-culture and nZVI over those that were abiotic containing nZVI. In the bottles that did contain PEI, however, this increase did not occur. This shows that enhancement due to the presence of co-culture with the nZVI was eliminated by the presence of PEI and that may be due to inhibitory impact of PEI. Also, it can be seen that the initial concentration of U(VI) in the bottles containing the PEI was lower than in those without PEI. This suggests that the PEI interacted with the U(VI) in a way that removed it from solution.

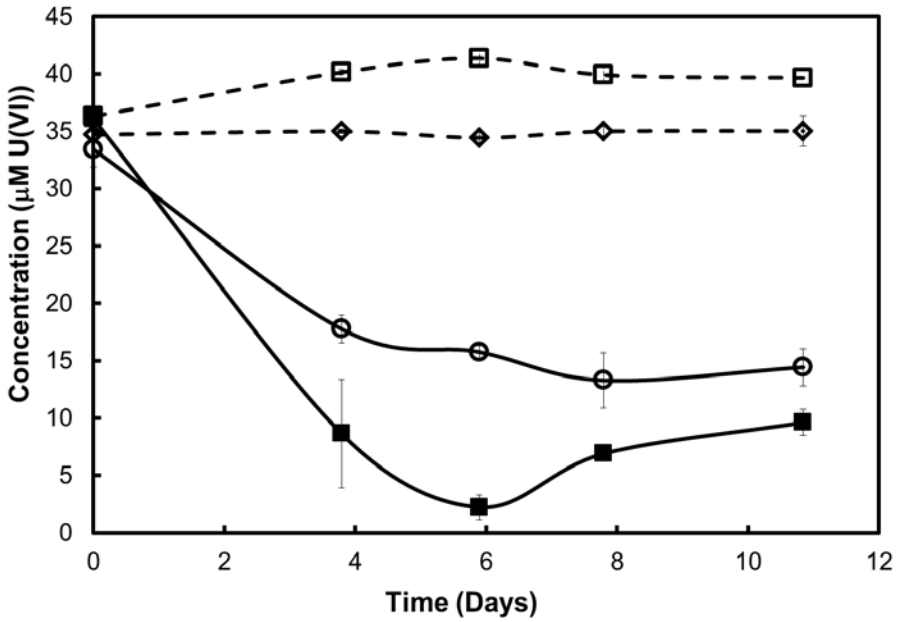


Fig. 5. Removal of soluble U(VI) by the microbial co-culture with nZVI without PEI. Legends: ---◊---, Abiotic, no Fe^0; ---□---, Biological, no Fe^0; —○—, Abiotic with nano-Fe^0; —■—, Biological with nano-Fe^0.

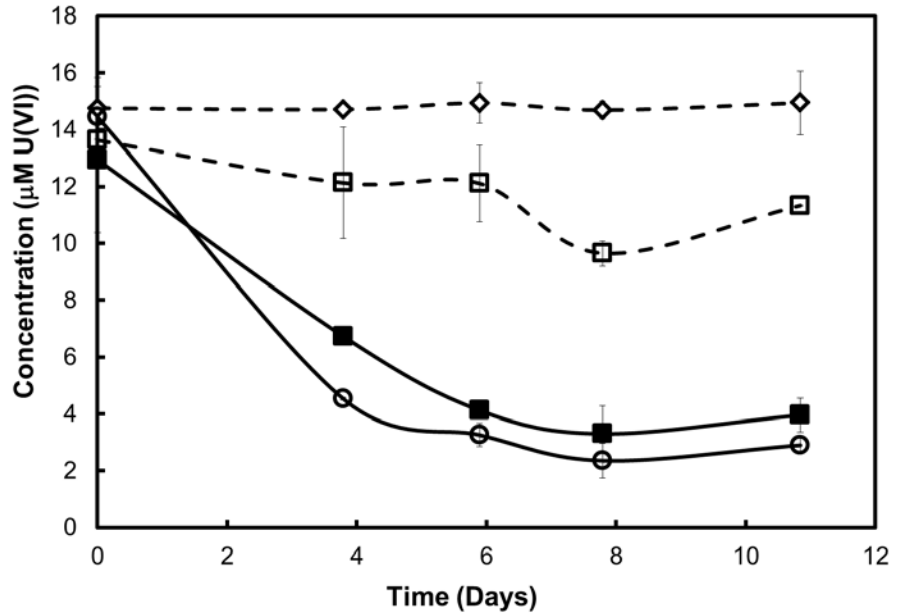


Fig. 6. Removal of soluble U(VI) by the microbial co-culture with nZVI with PEI. Legends: ---◊---, Abiotic with PEI, no Fe^0; ---□---, Biological with PEI, no Fe^0; —○—, Abiotic with nano-Fe^0 and PEI; —■—, Biological with nano-Fe^0 and PEI.

Conclusions

Results demonstrate that microbial co-culture enhanced the uranium reduction over the abiotic rate with ZVI as electron donor. This enhancement can occur whether the ZVI present is micron-sized or nano-sized. Furthermore, improvements in both the biological and abiotic rates of uranium removal rates were observed due to the use of nZVI compared to micron-sized ZVI. The rate improvement is most likely due to an improved surface area of the smaller particle size of nZVI. Polymeric surfactants, such as PEI, may improve the stability of nZVI nanoparticles in aqueous solutions. However, PEI interacts with the soluble uranium in an unknown way that results in the uranium being partially removed from solution. Future work should evaluate alternative dispersing agents which would work better in improving the stability of nZVI in aqueous solutions.

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