Evaluation of sustained release polylactate electron donors for removal of hexavalent chromium from contaminated groundwater

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Abstract

To evaluate the efficacy of bioimmobilization of Cr(VI) in groundwater at the Department of Energy Hanford site, we conducted a series of microcosm experiments using a range of commercial electron donors with varying degrees of lactate polymerization (polylactate). These experiments were conducted using Hanford Formation sediments (coarse sand and gravel) immersed in Hanford groundwater, which were amended with Cr(VI) and several types of lactate-based electron donors (Hydrogen Release Compound, HRC; primer-HRC, pHRC; extended release HRC, HRC-X) and the polylactate-cysteine form (Metal Remediation Compound, MRC). The results showed that polylactate compounds stimulated an increase in bacterial biomass and activity to a greater extent than sodium lactate when applied at equivalent carbon concentrations. At the same time, concentrations of headspace hydrogen and methane increased and correlated with changes in the microbial community structure. Enrichment of *Pseudomonas* spp. occurred with all lactate additions, and enrichment of sulfate-reducing *Desulfosporosinus* spp. occurred with almost complete sulfate reduction. The results of these experiments demonstrate that amendment with the pHRC and MRC forms result in effective removal of Cr(VI) from solution most likely by both direct (enzymatic) and indirect (microbially generated reductant) mechanisms.

Keywords: hexavalent chromium; bioremediation; bacteria; polylactate; metal reduction

1. Introduction

Due to its multiple uses in wood preservation, metal plating and corrosion inhibition, leather tanning and pigmentation, chromium is a frequent contaminant in soils and sediments (Riley et al., 1992). Fifty years of nuclear weapons production at the DOE Hanford reservation (Washington, USA) resulted in 1.7 trillion L of liquid waste being released to the soil and groundwater, resulting in chromium concentrations of up to 50 ppm (Hartman and Peterson, 2003) in the Hanford 100 Areas. Recent pump and treat applications have effectively reduced the Cr(VI) concentration below the drinking water standard (100 ppb) in all parts of the 100-H site and below 50 ppb in most areas with the exception of the area between the 100-H reactor and the Columbia River (USDOE, 2006). The US EPA ambient Water Quality Standard is currently set at 22 ppb and strategies are being evaluated to decrease Cr(VI) concentrations below this level before discharge to the Columbia River in order to protect salmon spawning beds (USDOE, 2006).

To reduce the concentration of Cr(VI) in groundwater, one promising strategy is its bioimmobilizaiton by means of reduction to the less mobile and less toxic form, Cr(III). This process may be accelerated by the stimulation of indigenous sediment and groundwater microorganisms through the addition of electron donors typically in the form of organic carbon (Cervantes, 1991; Ganguli and Tripathi, 1999; Viamajala et al., 2007; Faybishenko et al., 2008). Catalysis of Cr(VI) reduction by microorganisms may be directly mediated through direct electron transfer (Chardin et al., 2003; Ackerley et al., 2004; Cummings et al., 2007) or by indirect electron shuttling through metabolic by-products such as Fe(II) from iron reducing bacteria (Wielinga et al., 2001) or reactive

sulfides from sulfate reducing bacteria (Kim et al., 2001; Lan et al., 2005). In reality, a combination of these direct and indirect processes is expected (Tokunaga et al., 2003). Maintaining heavy metals in reduced and insoluble forms under field conditions may be challenging and can require regular application of organic carbon to maintain low concentrations of potential oxidants such as Mn (III, IV) (Tokunaga et al., 2007).

To increase the effectiveness of organic carbon application, several types of slow release compounds have been developed. Regenesis Inc. (San Clemente, CA) manufactures several slow release electron donor products based upon polymerized lactate. The released lactate may be subsequently fermented resulting in the production of hydrogen, hence these products are termed hydrogen release compounds (HRC). These products have been used successfully in accelerating bioremediation of trinitrotoluene (Barnes et al., 2001), chlorinated solvent contaminated sites, (Koenigsberg, 2001) and metal reduction (Willett and Koenigsberg, 2003). A range of HRC formulations are produced with varying viscosities and rates of lactic acid release from a glycerol tripolylactate complex. HRC-Primer is a faster lactate-releasing version of the original HRC with a glycerol tripolylactate content of 10% and viscosity of approximately 0.01 Pascals (water being 0.001); HRC is the original compound with a tripolylactate content of approximately 50%, a viscosity of approximately 20 Pascals centipoise is expected to promote aquifer reducing conditions for 24 months or longer. HRC-X is an extended release version of the original HRC compound that is expected to promote reducing conditions for up to 60 months, it has a viscosity of 200 Pascals and contains approximately 100% glycerol tripolylactate. MRC or metals remediation compound has similar properties to HRC but also contains sorbitol hexacysteinate, which

upon microbial degradation releases an organosulfur compound (cysteine). The organosulfur compound reacts to produce a reduced metal-organosulfur complex which precipitates or sorbs strongly to sediments (Regenesis, 2005).

In this microcosm study, we evaluated the application of these compounds for immobilization of Cr(VI) in groundwater resulting from the stimulation of microbial community activity and composition, using direct cell counts, enzyme activity assays and molecular tools, including 16S rRNA gene sequencing and terminal restriction fragment length polymorphism.

2. Materials and Methods

2.1. Microcosm Cr(VI) incubations.

The sediment material used for microcosm incubations was collected from the White Bluffs area within the Hanford Nuclear Reservation managed by the US DoE. The sample was collected from a surface-analog site that is representative of the Hanford formation within the 100 Area. Flasks with gas tight sampling ports were constructed, each containing 100 g Hanford formation surface sediment and 33 mL of groundwater from well 699-96-43. The groundwater used had a pH of 7.1 and contained 52(±1) ppb Cr(VI), 10(±0.7) ppm NO_3^--N, 86(±16) ppm sulfate, 29(±0.1) ppm dissolved inorganic carbon (DIC), 1.2 ppm dissolved organic carbon (DOC) and < 0.08 ppm NO_2^--N and PO_4^{2-}-P. Triplicate flasks were amended according to Table 1 and Cr(VI) concentrations were adjusted to a concentration of 1000 ppb. Electron donor in varying forms (40 mg carbon equivalent) was added to all flasks except for the "No Carbon" control. This

quantity of carbon relates to approximately 1 g HRC kg^{-1} sediment or to a solution phase carbon concentration of 100 mM. Oxygen concentrations in the flask headspace were reduced by sparging with argon for 1 min.

2.2 Aqueous phase chemical analyses.

pH, Cr(VI), DOC and H_2 were measured after set-up (3 h) and at weekly intervals up to 3 wk. Chromium reduction in the microcosm was determined by a colorimetric assay using S-diphenylcarbazide (DPC), a highly specific complexing agent for Cr(VI). Specifically, a 200 µL aliquot of centrifuged (14,000 *g*, 10 min) liquid from the microcosm was added to a cocktail consisting of 400 µL 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffer [pH 7], 33 µL 3M H_2SO_4, 327 ml dH_2O and 40 µL 0.25% DPC reagent prepared in acidified acetone (Mabbett and Macaskie, 2001). Absorbance was read at 540 nm and samples were compared to a standard curve of potassium dichromate prepared in chromate free Hanford groundwater to account for any matrix interference. DOC was analyzed using a TIC-TOC analyzer, (O-I Analytical, College Station, TX), while headspace gas concentrations were determined for CO_2, CH_4 and H_2 by gas chromatography. A subset of inorganic anions (nitrate, nitrite, phosphate, and sulfate) in the original groundwater and following 3 wk incubation was analyzed by ion chromatography.

2.3 Analysis of microbial activity and extraction of DNA from sediments.

Microbial dehydrogenase enzyme activity was determined by iodonitro-tetrazolium chloride (INT) reduction (von Mersi and Schinner, 1991) under anaerobic

conditions at room temperature. For analysis of changes in bacterial community structure and composition, DNA was extracted, however the use of coarse sediments for this study, made direct DNA extraction problematic and for this reason we used a modification of a cell concentration technique. 15 mL of cell release buffer containing 0.2 M NaCl, 50 mM Tris-HCl (pH 8.0), 0.05 M sodium pyrophosphate (pH 7.0) and 0.01 % w/v Tween 80 was added to approximately 40 g of coarse sediment in sterile 50 mL centrifuge tubes. To resuspend cells, tubes were shaken on a flat bed vortex at full speed for 5 min. The resulting homogenate was removed by pipette and the sediment was re-extracted with a further 15 mL of cell release buffer. Homogenates were combined and centrifuged at 14,900 *g* for 20 min at 4°C. DNA was extracted from exactly 500 mg (wet weight) of the resulting pellets using a BIO101 soil DNA extraction kit (QBiogene, Morgan Irvine, CA) according to the manufacturer's protocol.

2.4 PCR amplification of 16S ribosomal RNA genes (16S rDNA).

PCR was performed in triplicate and amplicons were pooled for each extract. PCR reactions were performed in a final volume of 100 μL and contained 1x Takara ExTaq PCR buffer, 2 mM $MgCl_2$, 300 μM primers (27F and 1492R), 200 μM deoxynucleotide triphosphates, 50 μg Bovine Serum Albumin, 0.5 μL of DNA extract and 2.5 U ExTaq DNA polymerase (Takara Mirus Bio, Madison, WI) and sterile milliQ H_2O added to bring the final volume up to 100 μL. For terminal restriction fragment length polymorphism (T-RFLP) analysis the forward primer 27F was labeled with 6-carboxyfluorescein for detection by capillary electrophoresis. Cycling was performed with initial denaturation at 95 °C (5 min) followed by 35 cycles of 95 °C (30s) 53 °C

(30s) 72 °C (1 min) and a final extension at 72 °C for 7 min. Amplicons were run on 1 % agarose gels pre-stained with ethidium bromide and viewed under UV transillumination. Amplicons were purified using an UltraClean PCR clean up kit (MoBio, Carlsbad, CA) according to the manufacturer's protocol, eluted in a final volume of 50 μL and quantified by gel electrophoresis prior to cloning and T-RFLP analysis.

2.5 Analysis of bacterial community composition and dynamics.

TRFLP analysis was carried out as described previously (Brodie et al., 2006) with Principal Component Analysis (PCA) performed using the software package PC-Ord v.4.01 (MjM Software, Gleneden Beach, OR). Regression of principal component axes against geochemical variables was carried out using packages in the R statistical software environment (R Development Core Team, 2007). For analysis of dominant bacterial taxa after destructive harvesting of microcosms, 16S rRNA gene amplicons were sequenced**.** Amplified rRNA genes were ligated to vector pCR2.1 and transformed into chemically competent *Escherichia coli* TOP10 cells according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Blue/white screening was carried out and white colonies were tested for inserts of correct size by colony PCR using vector primers M13 and M13(-20). PCR products were purified as described above and used as template for 5' end sequencing reactions using the BigDye v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) as recommended. Sequencing reactions were desalted prior to capillary sequencing on an ABI3100 Genetic Analyzer.

Sequencing chromatograms were analyzed for quality using the Phred base-caller (Ewing and Green, 1998) with a Phred quality score threshold of > q15. High quality

trimmed sequences were then screened for vector sequence using cross-match, (P. Green, http://bozeman.mbt.washing-ton.edu/phrap.docs/phrap.html). After trimming and vector removal, sequences with less than 400 base pairs of quality sequence (> q15) were discarded. Sequences were aligned to the greengenes 16S rRNA gene database (http://greengenes.lbl.gov) using the NAST tool (DeSantis et al., 2006) and were classified into bacterial families using the classify tool as described previously (Brodie et al., 2007; DeSantis et al., 2007). Sequences have been deposited in GenBank under pending accession numbers.

3. Results and Discussion

3.1 Influence of polylactate amendments on microcosm solution pH

Preliminary studies in deionized water without Hanford sediment or groundwater demonstrated reduced solution pH with all HRC products (data not shown), and this was also observed following amendment with all HRC formulations with decreases ranging 0.2 to 0.8 units relative to the no carbon control (Fig. 1a) however pH gradually increased in all incubations possibly as a result of nitrate reduction.

3.2 Removal of hexavalent chromium from solution

Figure 1b demonstrates that amendments of all slow-release compounds led to the decrease in the Cr(VI) concentration. HRC reduced Cr(VI) concentration below the detection limit (30 ppb) after 2 wk, this was also noted in a field application of HRC at the Hanford 100H site (Faybishenko et al., 2008), although application MRC resulted in the most rapid reduction of soluble Cr(VI) with concentrations being below detection limits after less than 1 week. The rapid initial decrease in Cr(VI) concentration after MRC addition may be principally abiotic. Autoclaved controls also showed a rapid initial decrease with almost half of the Cr(VI) being removed from solution within 3h (see Fig. S1a-f). In all incubations, following the initial decreases in Cr concentrations, the removal was accelerated by microbial activity as the autoclaved controls only showed minimal further decreases in soluble Cr (Fig. S1a-f). Surprisingly, of all the carbon amendments, flasks with sodium lactate demonstrated the slowest reduction of Cr(VI) in solution. Flasks without carbon added did show significant but slow Cr(VI) removal, suggesting natural attenuation processes may be possible at this site, however after 3 wk

more than one third of the Cr(VI) remained in solution. As with the autoclaved controls, sediment sorption of Cr(VI) cannot be ruled out as a mechanism of removal of soluble Cr.

3.3 Metabolism of polylactate compounds, hydrogen production and terminal electron accepting processes.

After 3 wk incubation 75 % of Na-lactate organic carbon remained in solution, while only 71 % of HRC-X, 58 % of pHRC and HRC, and 52 % of MRC organic carbon were still in solution (Fig. 1c). This may have been a result of either higher sorption to sediments or slower hydrolysis rates of the polymerized hydrogen release compounds, or lower rates of metabolism of the sodium lactate by the indigenous microbial species, therefore headspace hydrogen concentrations were measured to assay for microbial metabolism of the added carbon. Hydrogen headspace concentrations have been used with some success to indicate principal terminal electron accepting processes (Lovley and Goodwin, 1988; Marsh and McInerney, 2001), as dissolved hydrogen comes into equilibrium with gases within approximately 20 min, (Novelli et al., 1987). After 3 wk incubation, headspace H_2 concentrations were as predicted from the relative content of polymerized lactate in the HRC compounds, although the non-polymerized sodium lactate did not produce hydrogen to the same extent. Concentrations ranged from 0.15 ppm H_2 in the headspace of no carbon treatment to 3, 82, 110, 132, and 151 ppm for the lactate, HRC-X, HRC, MRC, and pHRC respectively (Fig. 1d). Using Henry's Law constants for H_2 it was possible to calculate concentrations of H_2 soluble in the aqueous phase. H_2 concentrations in solution ranged from 0.12 nM in the no carbon treatment to

3, 64, 86, 104, or 118 nM in the lactate, HRC-X, HRC, MRC, or primer-HRC treatments respectively and although some carbon mineralization was observed in the abiotic controls (Fig. S2a-f), this was significantly less than in assay microcosms and hydrogen concentrations in all autoclaved controls were typically 5 % or less of the corresponding assay microcosms. Aqueous H_2 concentrations of 7-10 nM have been associated with methanogenesis; 1-1.5 nM with sulfate reduction; 0.2 nM with Fe(III) reduction and < 0.05 nM with Mn(IV) or nitrate reduction (Lovley and Goodwin, 1988). Cr(VI) reduction can be mediated by direct microbial reduction (Ackerley et al., 2004) over a wide range of redox potentials (Smith and Gadd, 2000; Chardin et al., 2003; Tokunaga et al., 2003) and with H_2 as a primary electron donor (Battaglia-Brunet et al., 2002). Indirect reduction through by-products of anaerobic microbial respiration, particularly Fe(II) (Wielinga et al., 2001) or reactive sulfides (Kim et al., 2001), may both contribute to, and exceed, rates of direct microbial reduction (Wielinga et al., 2001). The week 3 aqueous phase H_2 concentrations calculated here predicted that nitrate and direct Cr(VI) reduction should occur in all treatments, while sulfate reduction should occur in all treatments except those without carbon. However, analysis of 3-week anion concentrations (Table 2) demonstrates that nitrate and sulfate reduction occurred in all treatments. Closer inspection of the H_2 concentrations shows that adequate H_2 concentrations (1.5 ppm in headspace or 1.94 nM in aqueous phase) for sulfate reduction were achieved after week 1 in microcosms without added carbon. Complete denitrification did not occur in microcosms without carbon as stoichiometric concentrations of nitrite accumulated possibly due to electron donor limitation. The calculated dissolved H_2 concentrations also suggested that methanogenesis would be expected, however concentrations were below

atmospheric methane concentrations (2 ppm) until week 3, when concentrations were of the order MRC (5.7 ppm) > primer HRC (4.8 ppm) > HRC (3.7 ppm) > Na-lactate (3.1 ppm) > HRC-X (2.9 ppm) > No added Carbon (2.5 ppm).

3.4 Stimulation of indigenous microorganisms

Analysis of direct microscopic cell counts and dehydrogenase activity indicated that all HRC products stimulated microbial populations and their activity, with most products resulting in a greater than 100 fold increase in bacterial numbers after 3 wk (Fig. 2a). Carbon application of all forms resulted in a larger proportion of living cells compared to no carbon additions (Fig. 2a), and this was reflected in microbial activity for most compounds. The highest microbial activity (dehydrogenase measured as μg INF formed from the INT substrate g^{-1} h^{-1}) was noted in the pHRC and MRC amended flasks (104 ± 17 and 152 ± 59 respectively), where activity was up to 250 times greater than following HRC or HRC-X treatments (0.53 ± 0.11 and 0.59 ± 0.18 respectively). While no detectable microbial activity was noted in microcosms without added carbon, it was surprising that the Na-lactate amendment resulted both in lower cell counts and non-detectable dehydrogenase activity; this was consistent with measurements of hydrogen concentration. Although not explicitly described in the formulations, all polylactate products studied here contain unspecified quantities of phosphate. The inclusion of this additional nutrient may explain the increased microbial activity, biomass and Cr(VI) reducing capacity in polylactate treated microcosms compared with sodium lactate.

3.5 Bacterial community structure and dominant species composition

We evaluated the relative changes in bacterial community structure between treatments using T-RFLP analysis of 16S rRNA genes (Fig. 2b). Plotting the first two principal components indicated a divergence in bacterial community structure between those with and without added carbon. This divergence was mostly along the first principal component (PC1). End-point (3 week) concentrations of H_2 and DOC were negatively correlated (R^2 = -0.59, $p<0.05$ and R^2 = -0.67, $p<0.01$ respectively) with PC1 (bacterial community structure) suggesting a relationship between rate of carbon release and bacterial composition, while Cr(VI) concentration (R^2 = 0.65, $p<0.01$) and pH (R^2 = 0.59, $p< 0.01$) were positively correlated with PC1. HRC-type carbon treatments were mostly separated by PC2 but no environmental data correlated with this axis. Dominant bacterial species were determined by sequencing clone libraries of 16S rRNA genes (Fig. 3). In microcosms without added carbon the bacterial community appeared to be adapted to the low nutrient environment, consisting mostly of oligotrophic α- and β-proteobacteria, some Bacteroidetes and a bacterial predator *Bacteriovorax*. Amendment with lactate of any form resulted in an increase in *Pseudomonas,* which accounted for up to 70% of clones in the case of HRC. In a previous study performed in the field at Hanford 100H, HRC injection also resulted in enrichment of *Pseudomonas* (Faybishenko et al., 2008). In addition to being nitrate reducers, *Pseudomonas* spp. have been shown to directly reduce Cr(VI) to Cr(III) by means of a soluble chromate reductase flavoprotein, ChrR (Gonzalez et al., 2003; Ackerley et al., 2004). Following the HRC injection at Hanford, a *Pseudomonas* strain with similarity to ***P. stutzeri*** was isolated and shown to reduce Cr(VI) under both aerobic and denitrifying conditions, however a ChrR homolog was not detected and Cr(VI) reduction was co-metabolic (Han et al., 2010). All

polylactate compounds stimulated homoacetogenic *Sporomusa*, while pHRC and MRC also stimulated spore forming sulfate reducers *Desulfosporosinus*. The enrichment of these organisms supports the observed decrease in both nitrate and sulfate concentrations noted in microcosms with added carbon after 3 wk.

Overall, this study has demonstrated the potential to stimulate indigenous microbial species present in the Hanford Formation sediments with multiple forms of sustained hydrogen release carbon compounds. We observed differences in bacterial community structure and composition related to carbon release rate and provide evidence for Cr(VI) reduction that may be catalyzed both directly and indirectly by bacteria in response to polylactate and MRC addition.

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References:

Ackerley, D.F., Gonzalez, C.F., Park, C.H., Blake, R., Keyhan, A., Matin, A., 2004. Chromate-reducing properties of soluble Flavoproteins from *Pseudomonas putida* and *Escherichia coli*. Appl. Environ. Microb. 70, 873-882.

Barnes, P.W., Heaston, M.S., Compton, J.C., 2001. Treatment of explosives-contaminated groundwater by in-situ cometabolic reduction. Sixth Annual In-Situ and On-Site Bioremediation Conference, San Diego, CA.

Battaglia-Brunet, F., Foucher, S., Denamur, A., Ignatiadis, I., Michel, C., Morin, D., 2002. Reduction of chromate by fixed films of sulfate-reducing bacteria using hydrogen as an electron source. J. Ind. Microbiol. Biotechnol. 28, 154-159.

Brodie, E.L., Desantis, T.Z., Joyner, D.C., Baek, S.M., Larsen, J.T., Andersen, G.L., Hazen, T.C., Richardson, P.M., Herman, D.J., Tokunaga, T.K., Wan, J.M., Firestone, M.K., 2006. Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. Appl. Environ. Microbiol. 72, 6288-6298.

Brodie, E.L., Desantis, T.Z., Parker, J.P., Zubietta, I.X., Piceno, Y.M., Andersen, G.L., 2007. Urban aerosols harbor diverse and dynamic bacterial populations. Proc. Natl. Acad. Sci. USA. 104, 299-304.

Cervantes, C., 1991. Bacterial interactions with chromate. Antonie Leeuwenhoek 59, 229-233.

Chardin, B., Giudici-Orticoni, M.T., De Luca, G., Guigliarelli, B., Bruschi, M., 2003. Hydrogenases in sulfate-reducing bacteria function as chromium reductase. Appl. Microbiol. Biotechnol. 63, 315-321.

Cummings, D.E., Fendorf, S., Singh, N., Sani, R.K., Peyton, B.M., Magnuson, T.S., 2007. Reduction of Cr(VI) under acidic conditions by the facultative Fe(lll)-reducing bacterium *Acidiphilium cryptum*. Environ. Sci. Technol. 41, 146-152.

DeSantis, T.Z., Brodie, E.L., Moberg, J.P., Zubietta, I.X., Piceno, Y.M., Andersen, G.L., 2007. High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. Microb. Ecol. 53, 371-383.

DeSantis, T.Z., Jr., Hugenholtz, P., Keller, K., Brodie, E.L., Larsen, N., Piceno, Y.M., Phan, R., Andersen, G.L., 2006. NAST: A multiple sequence alignment server for comparative analysis of 16S rRNA genes. Nucleic Acid Res. 34, W394-399.

Ewing, B., Green, P., 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res. 8, 186-194.

Faybishenko, B., Hazen, T.C., Long, P.E., Brodie, E.L., Conrad, M.E., Hubbard, S.S., Christensen, J.N., Joyner, D., Borglin, S.E., Chakraborty, R., Williams, K.H., Peterson, J.E., Chen, J., Brown, S.T., Tokunaga, T.K., Wan, J., Firestone, M., Newcomer, D.R., Resch, C.T., Cantrell, K.J., Willett, A., Koenigsberg, S., 2008. In situ long-term reductive nioimmobilization of Cr(VI) in groundwater using Hydrogen Release Compound. Environ. Sci. Technol. 42, 8478-8485.

Ganguli, A., Tripathi, A.K., 1999. Survival and chromate reducing ability of *Pseudomonas aeruginosa* in industrial effluents. Lett. Appl. Microbiol. 28, 76-80.

Gonzalez, C.F., Ackerley, D.F., Park, C.H., Matin, A., 2003. A soluble flavoprotein contributes to chromate reduction and tolerance by *Pseudomonas putida*. Acta Biotechnol. 23, 233-239.

Han, R., Geller, J.T., Yang, L., Brodie, E.L., Chakraborty, R., Larsen, J.T., Beller, H.R. 2010. Physiological and transcriptional studies of Cr(VI) reduction under aerobic and denitrifying conditions by an aquifer-derived Pseudomonad. Environ. Sci. Technol. 44, 7491-7497.

Hartman, M.J., Peterson, R.E., 2003. Aquifer Sampling Tube Results for Fiscal Year 2003. Pacific Northwest National Laboratory report number PNNL-14444, Richland, WA.

Kim, C., Zhou, Q., Deng, B., Thornton, E.C., Xu, H., 2001. Chromium(VI) reduction by hydrogen sulfide in aqueous media: Stoichiometry and kinetics. Environ. Sci. Technol. 35, 2219-2225.

Koenigsberg, S.S., 2001. Results from forty-two field applications of Hydrogen Release Compound for accelerated bioattenuation. Abstr. Am. Chem. Soc. 221, U464-U465.

Lan, Y., Deng, B., Kim, C., Thornton, E.C., Xu, H., 2005. Catalysis of elemental sulfur nanoparticles on chromium(VI) reduction by sulfide under anaerobic conditions. Environ. Sci. Technol. 39, 2087-2094.

Lovley, D.R., Goodwin, S., 1988. Hydrogen concentrations as an indicator of the predominant terminal electron-accepting reactions in aquatic sediments. Geochim. Cosmochim. Ac. 52, 2993-3003.

Mabbett, A., Macaskie, L., 2001. A novel isolate of *Desulfovibrio* sp. with enhanced ability to reduce Cr(VI). Biotechnol. Letts. 23, 683-687.

Marsh, T.L., McInerney, M.J., 2001. Relationship of hydrogen bioavailability to chromate reduction in aquifer sediments. Appl. Environ. Microbiol. 67, 1517-1521.

Novelli, P.C., Scranton, M.I., Michener, R.H., 1987. Hydrogen distributions in marine sediments. Limnol. Oceanogr. 32, 565-576.

R Development Core Team, 2007. R: A Language and Environment for Statistical Computing. Vienna, Austria. R Foundation for Statistical Computing,Vienna, Austria. http://www.R-project.org.

Regenesis, 2005. Metal Remediating Compound. http://www.regenesis.com/contaminated-site-remediation-products/chromium-immobilization/chroImm/

Riley, R.G., Zachara, J.M., Wobber, F.J., 1992. Chemical contaminants on DOE lands and selection of contaminant mixtures for subsurface science research [microform]. R.G. Riley, J.M. Zachara in collaboration with F.J. Wobber U.S. Dept. of Energy, Office of Energy Research, Subsurface Science Program; Available to the public from the National Technical Information Service, U.S. Dept. of Commerce, Washington, D.C.

Smith, W.L., Gadd, G.M., 2000. Reduction and precipitation of chromate by mixed culture sulphate-reducing bacterial biofilms. J. Appl. Microbiol. 88, 983-991.

Tokunaga, T.K., Wan, J., Lanzirotti, A., Sutton, S.R., Newville, M., Rao, W., 2007. Long-term stability of organic carbon-stimulated chromate reduction in contaminated soils and its relation to manganese redox status. Environ. Sci. Technol. 41, 4326-4331.

Tokunaga, T.K., Wan, J.M., Firestone, M.K., Hazen, T.C., Olson, K.R., Herman, D.J., Sutton, S.R., Lanzirotti, A., 2003. In situ reduction of chromium(VI) in heavily contaminated soils through organic carbon amendment. J. Environ. Qual. 32, 1641-1649.

USDOE, 2006. Hanford Groundwater Remediation Project. http://www.hanford.gov/page.cfm/SoilGroundwater.

Viamajala, S., Smith, W.A., Sam, R.K., Apel, W.A., Petersen, J.N., Neal, A.L., Roberto, F.F., Newby, D.T., Peyton, B.M., 2007. Isolation and characterization of Cr(VI) reducing *Cellulomonas* spp. from subsurface soils: Implications for long-term chromate reduction. Bioresource Technol. 98, 612-622.

von Mersi, W., Schinner, F., 1991. An improved and accurate method for determining the dehydrogenase activity of soils with iodonitrotetrazolium chloride. Biol. Fert. Soil. 11, 216-220.

Wielinga, B., Mizuba, M.M., Hansel, C.M., Fendorf, S., 2001. Iron promoted reduction of chromate by dissimilatory iron-reducing bacteria. Environ. Sci. Technol. 35, 522-527.

Willett, A., Koenigsberg, S.S., 2003. Metals remediation compound (MRC): A new slow release product for in situ metals remediation. Abstr. Am. Chem. Soc. 226, U590-U590.

Table captions.

Table 1. Batch simulation of Cr(VI) bioreduction. Triplicate flasks containing sediment and groundwater were amended with lactate containing compounds and Cr(VI). [a]Carbon content (w/w) of HRC/MRC products was determined for each lot received using a Carlo-Erba automated C/N analyzer.

Table 2. Anion analysis of aqueous solutions from microcosms after 3-week (504 h) incubation period. Values represent single analyses of pooled samples (n = 3).

Figure captions

Figure 1. Analysis of microcosm solution and headspace over time following amendment with organic carbon compounds. **(A)** Solution pH; **(B)** Cr(VI) concentration (ppb); **(C)** Solution phase DOC (ppm). **(D)** Headspace H_2 concentration (ppm). Data represents mean ± standard error (n = 3).

Figure 2. (A) Epifluorescent bacterial cell counts in microcosm sediment samples following 3 wk incubation in the presence of organic carbon compounds and Cr(VI). Values represent mean of triplicate analyses. **(B)** Principal components analysis of T-RFLP-based bacterial community composition of microcosm sediment samples following 3 wk incubation in the presence of hydrogen release carbon compounds and Cr(VI). Data represents mean ± standard error (n = 3).

Figure 3. Sequence distribution in clone libraries of 16S rDNA genes amplified from microcosms incubated in the presence of organic carbon compounds and Cr(VI).

Numbers in pie slices indicate closest phylogenetic match. (1) *Methylophilus*, (2) *Sphingobacterium*, (3) *Oxalobacter*, (4) *Arthrobacter*, (5) *Bacteriovorax*, (6) *Mesorhizobium*, (7) *Ramlibacter*, (8) *Chitinophaga*, (9) unclassified *Burkholderiales*, (10) *Dechloromonas*, (11) *Pseudomonas*, (12) *Rhizobium*, (13) *Azoarcus*, (14) *Sporomusa*, (15) *Acidovorax*, (16) *Sinorhizobium*, (17) *Desulfosporosinus*, (18) unclassified gamma proteobacteria, (19) *Variovorax*, (20) *Vogesella*, (21) *Rhodoferax*, (22) *Clostridium*, (23) *Serratia*.

Figure S1. Aqueous phase Cr(VI) concentrations (ppb) in microcosms incubated with (A) No added carbon; (B) Lactate; (C) pHRC; (D) HRC; (E) HRC-X and (F) MRC. Continuous lines with closed circles show assay microcosms and dashed lines with open circles show sterile controls.

Figure S2. Microcosm headspace CO_2 and H_2 concentrations (ppm) in microcosms incubated with (A) No added carbon; (B) Lactate; (C) pHRC; (D) HRC; (E) HRC-X and (F) MRC. For CO_2 data, continuous lines with closed circles show assay microcosms and dashed lines with open circles show sterile controls. For H_2 data continuous lines with closed squares show assay microcosms and dashed lines with open squares show sterile controls.

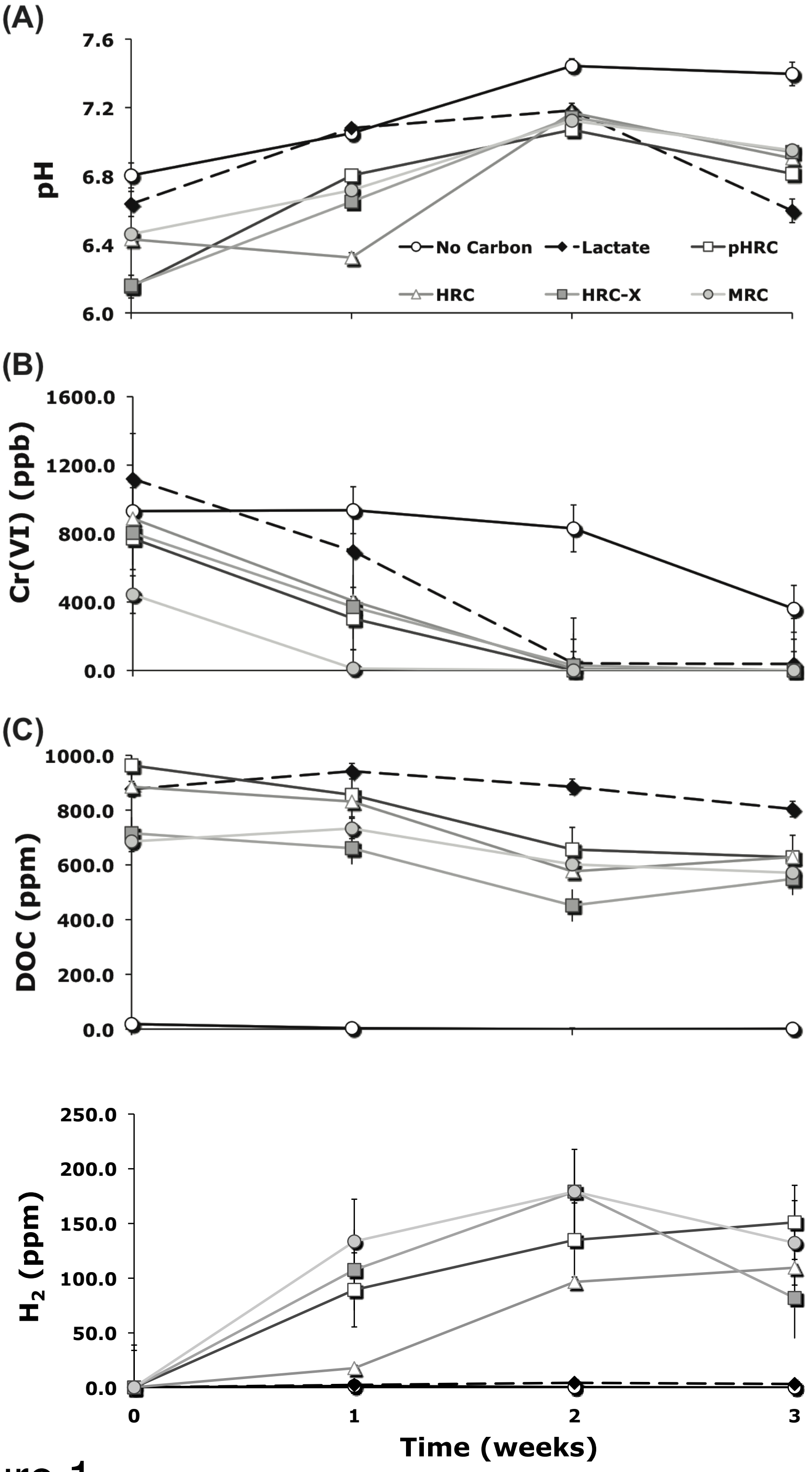
(A)
pH
7.6
7.2
6.8
6.4
6.0
No Carbon
Lactate
pHRC
HRC
HRC-X
MRC
(B)
Cr(VI) (ppb)
1600.0
1200.0
800.0
400.0
0.0
(C)
DOC (ppm)
1000.0
800.0
600.0
400.0
200.0
0.0
H2 (ppm)
250.0
200.0
150.0
100.0
50.0
0.0
0
1
2
3
Time (weeks)

Figure 1

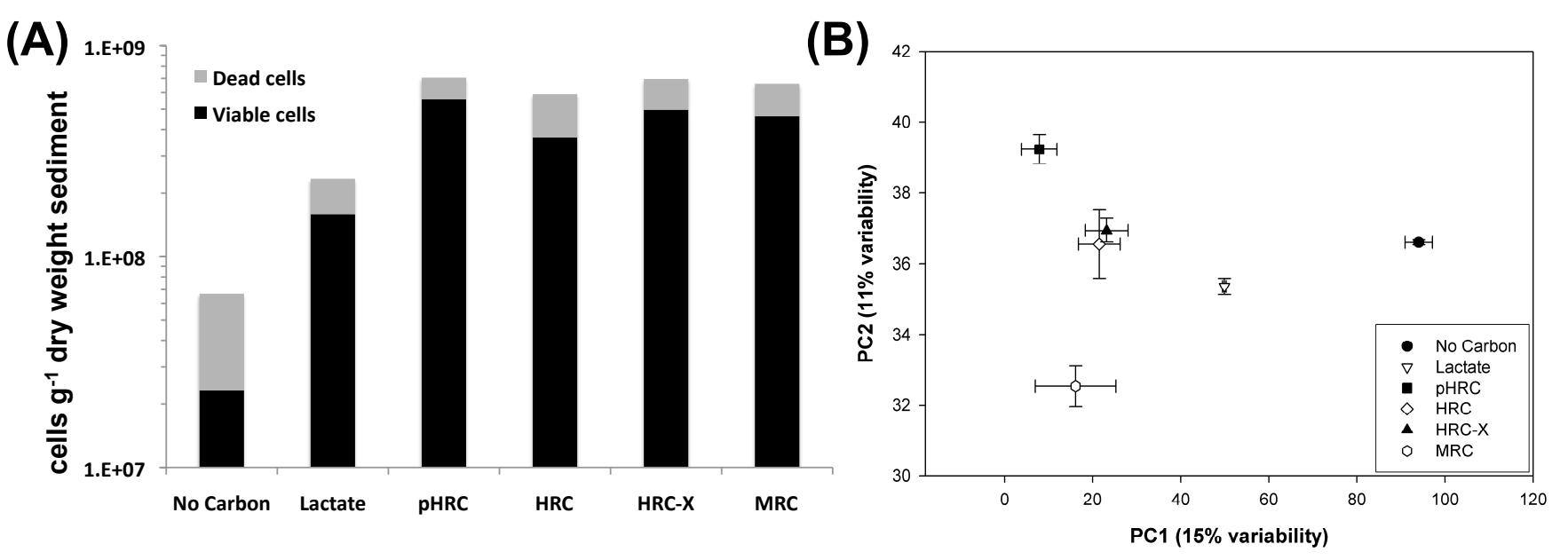
(A)
1.E+09
1.E+08
1.E+07
cells g-1 dry weight sediment
Dead cells
Viable cells
No Carbon
Lactate
pHRC
HRC
HRC-X
MRC
(B)
42
40
38
36
34
32
30
PC2 (11% variability)
0
20
40
60
80
100
120
PC1 (15% variability)
No Carbon
Lactate
pHRC
HRC
HRC-X
MRC

Figure 2.

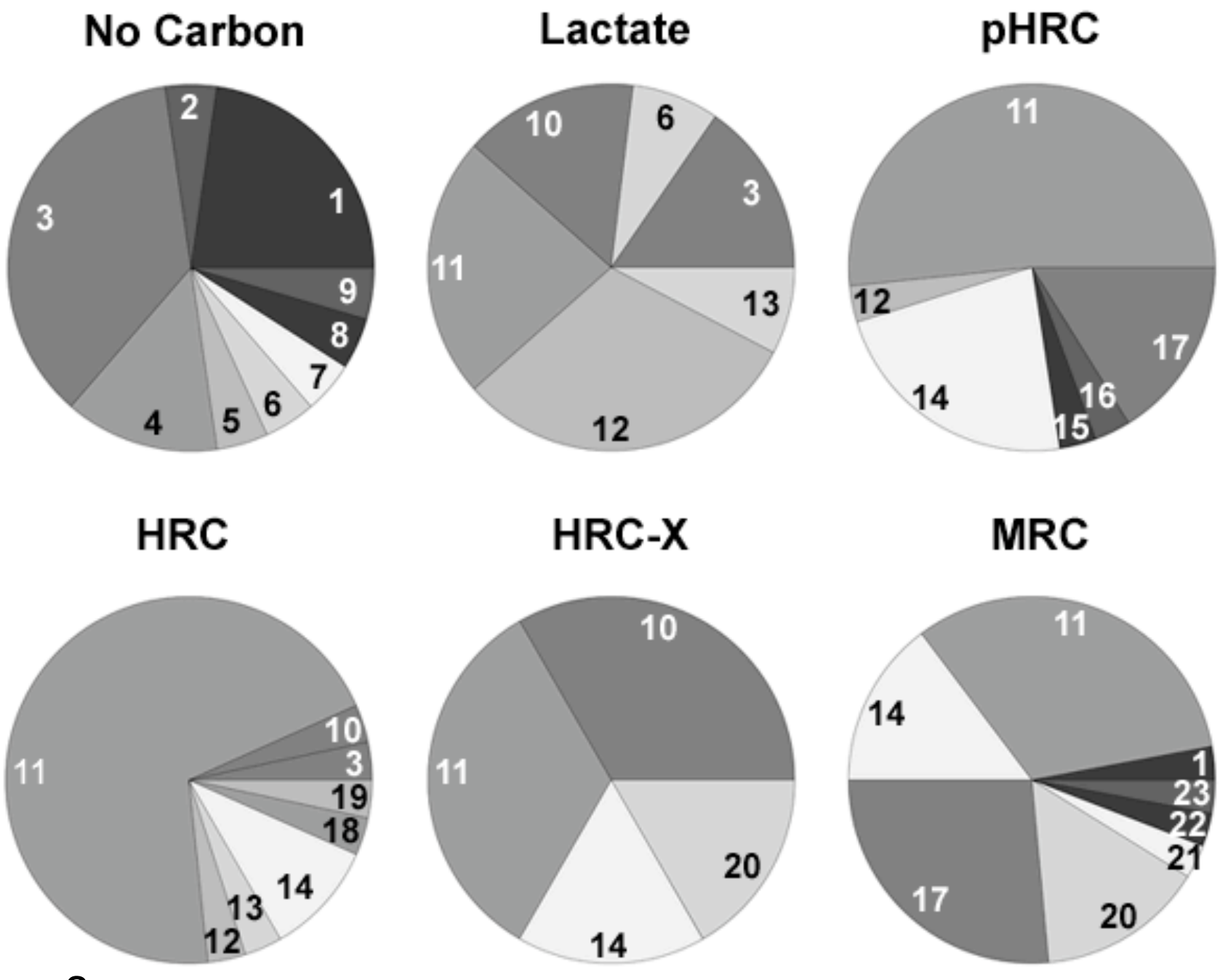
No Carbon
2
1
3
9
8
7
6
5
4
Lactate
10
6
3
11
13
12
pHRC
11
12
17
14
16
15
HRC
11
10
3
19
18
14
13
12
HRC-X
10
11
20
14
MRC
11
14
1
23
22
21
17
20

Figure 3

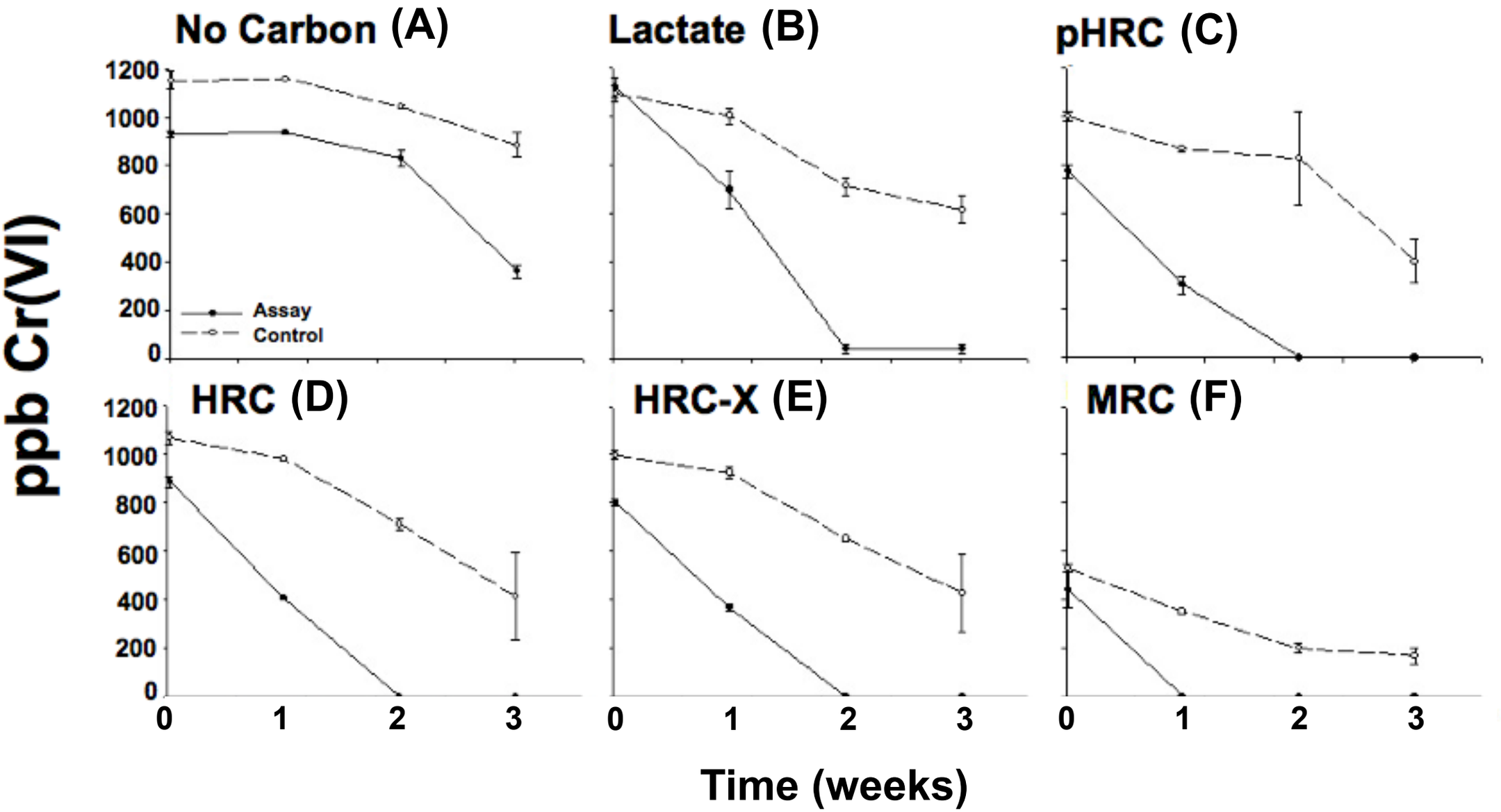
No Carbon (A)
Lactate (B)
pHRC (C)
HRC (D)
HRC-X (E)
MRC (F)
Cr(VI)
ppb
1200
1000
800
600
400
200
0
Assay
Control
0
1
2
3
Time (weeks)

Figure S1

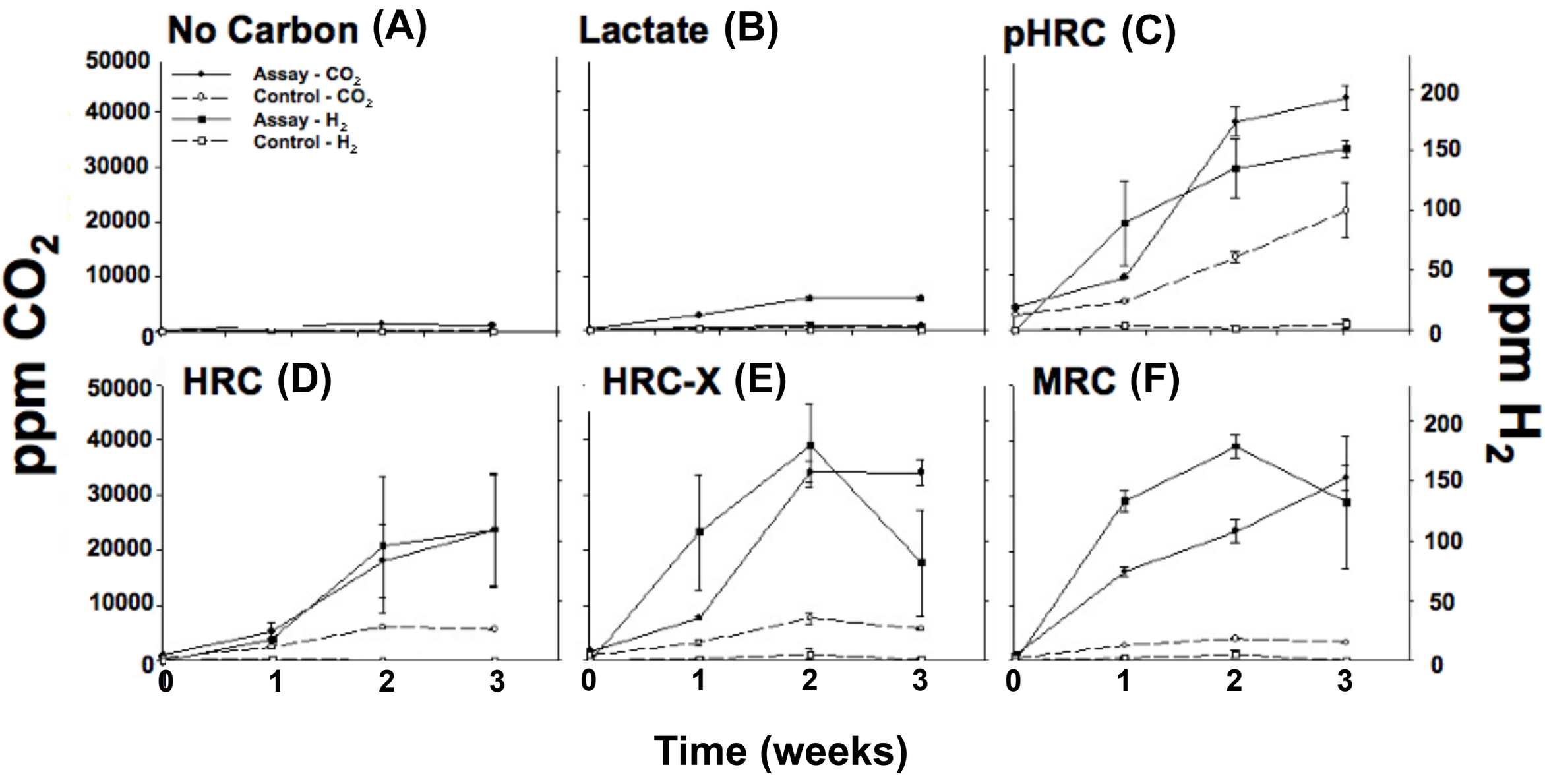
No Carbon (A)
Lactate (B)
pHRC (C)
HRC (D)
HRC-X (E)
MRC (F)
Assay - CO2
Control - CO2
Assay - H2
Control - H2
ppm CO2
ppm H2
Time (weeks)
50000
40000
30000
20000
10000
0
200
150
100
50
0
1
2
3

Figure S2