Microbial stabilization of Plutonium in the Subsurface Environment

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I. Overview.

This report outlines the results of work performed at the Colorado School of Mines, Brookhaven National Laboratory and Texas A&M University during the second reporting phase of this project (approximately 2.5 years of project duration).

The sub-projects focused on this year include:

- A. Biotransformation of Pu-contaminated soil
- B. Environmental colloids at the Rocky Flats Environmental Technology Site
- C. Production, isolation and characterization of EPS (exopolymeric substances, or: exopolysaccharides).
- D. Colloid trapping
- E. Determination of stability constants of complexes of Pu(IV) with organic ligands.
- F. The role of bacterial EPS in the transport of Pu through saturated porous media.

II. Details of Individual sub-Projects.

Our current NABIR project included elements on: isoelectric focusing experiments to determine the oxidation state, molecular weight, and association of Pu with organic and mineral matter from contaminated soils in Colorado; the isolation, separation, purification and characterization of polysaccharide-rich EPS from bacteria cultures for use in biotransformation and complexation experiments; evaluation of Pu complex formation with environmentally relevant organic ligands, including bacterial exudates and the development of methods for determining the stability constants for Pu with polyprotic ligands; the biotransformation of Pu in contaminated soils; Pu oxidation state determination; and Pu / citrate biodegradation. Highlights are summarized below; some results are presented in the annual NABIR reports for this project.

Current results progressed as follows: Microbial transformation of RFETS soil → Isolation of the predominant colloidal Pu(IV) species in RFETS soil → Isolation of EPS from NABIR-relevant microorganisms → Interaction of Pu(IV)-EPS with minerals → Understanding the thermodynamic stability of Pu(IV) in association with microbial ligands. These results lead us to focus the current proposal on gaining a detailed understanding of the biogeochemistry of Pu(IV) colloids (inorganic and organic) with emphasis on attenuation processes for these mobile phases.

2.1 Biotransformation of Pu in Contaminated Soil. Microbial activity can affect the stability and the mobility of Pu in soils and sediments over the long-term in the following ways: (i) dissolution due to production of organic acids and sequestering agents, (ii) reductive dissolution of iron and release of Pu associated with iron oxides, (iii) immobilization due to precipitation

reactions and by biomass/cellular polymeric substances and exopolymers, and (iv) biodegradation of the organic carbon associated with Pu fractions. We examined the influence of anaerobic microbial activity on Pu mobility in Pu contaminated soil from the Rocky Flats Environmental Technology Site (RFETS). Two soils from the 903 Pad lip area, the source-term for the contaminated soils and ponds at RFETS, were used with total $^{239,240}\text{Pu}$ content of 246 pCi g^{-1} (4 ppb Pu, soil sample 1) and 78 pCi g^{-1} (1 ppb Pu, soil sample 3). The sequential selective extraction protocol of Yong *et al.* (1993) was used with modification to obtain information regarding the mineralogical distribution of Pu in the soil sample. The majority (60%) of the total Pu in the soil was found to reside in the inert fraction (HF and HClO_4 digestion) of sample 1, while the majority (80%) was found to reside in the organic fraction ($\text{H}_2\text{O}_2/\text{NH}_4\text{OAc}$ digestion) of soil 3. In addition, 2.2% and 1.9% of the Pu resided in the Fe and Mn oxide fraction of sample 1 and 3, respectively.

Five grams of the soil were prepared under anaerobic conditions in 125 ml serum bottles, stoppered with thick butyl-rubber stoppers, with 75 ml of the following amendments: i) pre-reduced (boiled and N_2 -purged), DI H_2O (no-amendment); ii) pre-reduced DI H_2O w/ 0.5% w/v glucose, 0.015% w/v NH_4Cl , and iii) pre-reduced DI H_2O w/ 0.5% w/v sodium lactate, 0.015% w/v NH_4Cl . Supernatant samples were monitored over 45 days for pH, Fe^{2+} and Fe^{3+} , organic acids, and at 45 days $^{239,240}\text{Pu}$ in the unfiltered supernatant was determined by alpha spectroscopy.

Figure 2 shows the dissolution of iron from the two soils as a result of microbial activity after 45 days. Figure 3 shows the concentration of Pu detected in the unfiltered supernatant at the same time period. The glucose treatment mobilized Pu from Soil 1 to the greatest extent (10 pCi g^{-1} dry weight or 4.1% of the total activity) as well as iron; Pu in this soil resides in the inert fraction but may be accessible in the iron-oxide fraction. Soil 3 showed little mobilization of Pu above that which was detected in the water treatment indicating that the Pu associated with the organic fraction may be more stable. Lactate had little effect on iron or Pu mobilization. These results show that fermentative microbial activity can mobilize Pu as Pu colloids in contaminated soil possibly due to dissolution of iron phases. The identity of the bacterially-mobilized Pu species is under investigation.

Figure 2. Dissolution of iron was greatest in Soil 1 incubated with glucose; lesser amounts of iron were dissolved from lactate amended samples.

Figure 3. Pu was mobilized in the unfiltered supernatant of soil 1 after 45 days with glucose amendment while less Pu was mobilized in soil 3.

2.2 Environmental colloids at the Rocky Flats Environmental Technology Site (RFETS): Santschi *et al.* (2002 a,b) show that Pu mobility at the RFETS site is controlled by in the presence of iron-enriched humic acid or by acid polysaccharide colloids that had been identified in RFETS soils and surface waters. In this study, a Pu(IV) containing 10 kDa organic-rich colloid with pH_{IEP} of around 3 was separated from soils. Furthermore, TEM (transmission electron microscopy) and chemical analysis of surface groundwater colloids from the Rocky Flats soils demonstrated that there were two types of organic colloids in these groundwaters: larger semi-crystalline cellulose-type degradation products, and smaller humic-type aggregates (Roberts *et al.*, 2004). Chemical analysis of these organic-rich colloids demonstrated substantial gradients of Pu/OC and Fe/OC ratios (OC = organic carbon) from surface waters to groundwaters at this site, suggesting a close coupling of the fate of Pu to that of organic carbon and iron in these environments. Phosphate was found to greatly diminish the mobility of total Pu and reduced particle residence times in holding ponds (Roberts and Santschi, 2004b).

2.3 Production, isolation and characterization of EPS (exopolymeric substances, or: exopolysaccharides): EPS were extracted from four bacteria species cultured in the lab: a) two aerobic soil bacteria: *Pseudomonas fluorescens* Biovar II and *Pseudomonas fluorescens*; b) one anaerobic bacterium, *Clostridium* sp. BC1 and c) a facultative iron-reducer: *Shewanella putrefaciens*. After testing a number of different published extraction procedures the results of which strongly depend on the subsequent use and application of EPS, we eventually settled on a procedure that separates EPS that are in solution from those that initially stay attached through ultracentrifugation. Those in solution were further separated and purified through ethanol precipitation, addition of NaCl to help in the isolation process and the addition of proteinase to degrade protein compounds in the nutrient broth and bacterial EPS samples, as well as subsequent dialysis and lyophilization. The EPS from the particulate (capsular) fraction was separated through resuspension, ultracentrifugation, and ethanol precipitation in the presence of

NaCl and subsequent dialysis and lyophilization. The final product was reprocessed, after initial filtration, through subsequent alcohol precipitation steps in a clean-room. Chemical purity of the different batches of each species and each fraction was verified through chemical analysis, which indicate that EPS-carbon consists 40-60% of total carbohydrates, 5-20% of proteins, 3-20% of uronic acids (which make up 10-30% of total carbohydrates). Galacturonic acid was a principal constituent of the EPS, as shown by GC-MS analysis. Physical purity was verified through TEM analysis (Fig. 4). Each batch produced 5-10 mg of freeze-dried material. Further chemical characterization through GC-MS analyses is in progress.

Fig. 4. TEM picture of dissolved EPS from *Shewanella putrefaciens*. Results from particulate fractions appear similar.

2.4 'Colloid trapping'. Preliminary experiments on Pu(IV) sorption to colloidal silica (with different pore size and surface area) in the presence or absence of model acid polysaccharides, as well as EPS harvested from soil bacteria (e.g., three aerobic soil bacteria: *Shewanella putrefaciens* CN32, *Pseudomonas fluorescens* Biovar II, and *Pseudomonas fluorescens*; and one anaerobic bacterium: *Clostridium* sp. BC1) suggest that microbially produced EPS can act as a colloid trap through a combination of steric, hydrophobic and hydrophilic interactions (Roberts *et al.*, 2004c). Colloid trapping by mineral particles resulted in an enhancement of the K_d value for Pu onto mineral particles such as silica (Santschi *et al.*, 2004), as compared to the linear sum of the individual K_d values (see Fig. 5).

2.5 Determination of stability constants of complexes of Pu(IV) with organic ligands. Here we focused on the complexation of Pu with the lipopolysaccharide (LPS) exocellular polymeric substances LPS-EPS component of natural organic matter. Much of EPS is composed of exopolysaccharides that are hydrophilic macromolecules consisting of monosaccharide units; as such, LPS-EPS are highly dispersed and complex molecules with a substantial polyfunctional, and polyelectrolytic behavior (Stefansson, 1999; Selmann and Holst, 2001).

Ternary System: Actinide-SiO₂-EPS

Fig. 5. a) Methodology (left) and b) preliminary results (right) from Pu(IV) sorption experiments in the ternary system: Pu(IV), SiO₂, and EPS, showing the enhancement effect in K_d values as compared to predictions based on endmember values.

There are two common LPSEPS's: cellulose and starch, both of which are derived from plants (Murphy, 2000). Microorganisms can also produce LPS-EPS; it is located in the cell wall of bacteria or outside of the cell surface (Seltmann and Holst, 2001). Due to the strong tendency of acid polysaccharides for complexation with metals, LPS-EPS have been used for the removal of toxic metals from wastewater (Fukushima *et al.*, 1999). Complexation of metal ions with polysaccharides is not fully understood but is believed to occur through carboxylic groups (Fane *et al.*, 1992). The requirement for establishing and maintaining direct-contact between dissimilatory iron reducing bacteria and iron oxides, so that metal reductases can function effectively, is accomplished by LPS-EPS (Korenevsky *et al.*, 2002).

Pu / organic ligand stability constants were determined through the application of a ligand competition method utilizing cation exchange resins. Details of the procedure can be found in Lenhart *et al.* (2000). Stability constants have often been estimated using Schubert's method; Schubert's method is a linearization technique used to analyze ion-exchange data. Lenhart *et al.* (2000) provides a detailed explanation of Schubert method (Schubert, 1948). Schubert's method, however, has limited applicability. As a result, a new data evaluation method that utilizes FITEQL (Herbelin and Westall, 1996) is being developed by our research NABIR group to estimate stability constants for complex, polyprotic macromolecules. FITEQL is a computer program that can be used to determine "best-fit" equilibrium constants for reactions that are postulated to explain experimental data.

Stability constants for Pu binding with the following ligands have been determined thus far: citric acid (to validate the procedure), galacturonic and alginic acids, and EPS from the bacteria *Clostridium* sp., *Pseudomonas fluorescens* and *Shewanella putrefaciens*.

Galacturonic acid is a principle component (40-60%) of pectin, a polysaccharide found in plants (Narkhede *et al.* 1994). Galacturonic acid has also been identified as part of this research as a predominant exudate (> 1 kD) produced by *Clostridium* sp. Alginic acid was used as a surrogate for the polysaccharide component of NOM. Alginic acids are a family of hydrophilic, colloidal polysaccharides commercially obtained from brown seaweeds (Stefansson, 1999). Bacterially-produced alginate is commonly present in soil environments due to production by N₂-fixing bacteria of the genus *Azotobacter* and has been studied for its ability to stabilize biofilms and protect bacteria of the genus *Pseudomonas* from antimicrobial agents (Sabra *et al.*,

2000; Brown *et al.*, 1995; Hanlon *et al.*, 2001). Alginate is an unbranched, binary copolymer composed of varying proportions of β -D mannuronic acid and α -L-guluronic acid linked through the 1- and 4- positions. Mannuronic and guluronic were also identified as predominant components of *Clostridia* sp.

Proton binding to organic macromolecules is 'localized'. However, most descriptions of proton binding to natural organic ligands are based on macroscopic, delocalized binding constructs. Affinity distribution models (e.g., Westall *et al.*, 1995) are centered on the idea that it is possible to describe the titration behavior of a complex molecule as a mixture of simple monoprotic acids or bases. The actual nature of the distributions derived from a titration curve can be quite varied (e.g., smooth or discrete) but generally the range of distributions provides good fits to the titration data. Whatever the goodness of fit, however, the affinity distributions provide little insight to the molecular-level mechanisms; however, they provide mathematical tractability for use in computer speciation codes.

Figure 6 shows the distributions of pK_a values determined from inverse modeling of alginic acid Potentiometric titration data. In this scheme, pK_a values are arbitrarily picked (e.g., 2, 4, etc.) and the site specific concentrations (mmol g^{-1}) are inverse modeled. The ligands with pK_a values of 2 and 4 may be considered to be representing carboxylic acids.

Figure 6. Graphical representation of the distribution of alginic acid and *Pseudomonas fluorescens* EPS 'ligands' using the discrete ligand affinity distribution approach. Bacterially-produced alginate is commonly present in soil environments due to production by N_2 -fixing bacteria of the genus *Azotobacter*.

Figure 7. Comparison of Pu (IV) association with EPS from the target bacteria *Clostridium* sp., *Pseudomonas fluorescens* and *Shewanella putrefaciens*. Note: for comparison, the K_d for Pu binding to alginic acid is $\sim 10^5 \text{ L / kg}$. Because the *Pseudomonas* has fewer acid groups, (per mass) relative to the alginic acid, the *Pseudomonas* Pu binding sites must be proportionately much stronger.

Actinide binding to the polyprotic ligands is also localized at the molecular level. However, simulations of the environmental fate of actinide / organic ligands complexes also requires mathematical tractability. Actinide / organic ligands binding models can be built upon the affinity distributions for proton binding. While also not 'correct' with respect to molecular-level mechanisms, such continuum level models are able to capture the macroscopic behavior over a

range of system conditions. Table 1 presents postulated formation reactions and the derived stability constants for some of the organic ligands evaluated. Figure 7 compares 'domains' of Pu (IV) association with the bacterially-derived EPS in terms of $\log K_d$ values. Note that there are substantial and significant differences between the extracellular materials.

2.6 Role of bacterial EPS in Pu transport through saturated sand. Preliminary transport experiments of Pu with and without *Pseudomonas fluorescense*-derived EPS indicates that EPS acts to 'solubilize' Pu under advective flow conditions. In the absence of EPS, Pu sorbs with irreversible first-order kinetics to an immobile phase. In contrast, Pu / EPS complexes injected into the transport column sorb reversibly and with a retardation factor of ~ 2 relative to the transport of a conservative tracer (HTO). Such behavior is consistent with the observations of Wildung *et al.* (1987) on the solubilization potential of bacterial exudates.

Table 1. Examples of postulated reactions and stability constants determined for environmental organic ligands.

Ligand	Postulated reaction	Log K_{ij} (I = 0)
Citrate	$\text{Pu}^{4+} + \text{L}^{3-} = \text{PuL}^+$	19.65
Galacturonic acid	$\text{Pu}^{4+} + \text{L}^- = \text{PuL}^{3+}$	15.32
Alginic acid ^a	$\text{Pu}^{4+} + 2\text{HL}_1 = \text{Pu}(\text{L}_1)_2^{2+} + 2\text{H}^+$	10.79
	$\text{Pu}^{4+} + 2\text{HL}_2 = \text{Pu}(\text{L}_2)_2^{2+} + 2\text{H}^+$	9.54
<i>Pseudomonas</i> EPS	$\text{Pu}^{4+} + 2\text{HL}_1 = \text{Pu}(\text{L}_1)_2^{2+} + 2\text{H}^+$	11.8
	$\text{Pu}^{4+} + 2\text{HL}_2 = \text{Pu}(\text{L}_2)_2^{2+} + 2\text{H}^+$	15.2
	$\text{Pu}^{4+} + 2\text{HL}_3 = \text{Pu}(\text{L}_3)_2^{2+} + 2\text{H}^+$	14.7

^a L_1 , L_2 and L_3 represent the $\text{pK}_a = 2, 4$ and 6 ligands, respectively.

Publications in preparation or submitted at this point from this work:

- Gillow, J.B., Honeyman, B.D., Francis, A.J. Anaerobic microbial transformation of plutonium in contaminated soil [to be submitted to *Radiochim. Acta*].
- Harper-Arabie, R., B.D. Honeyman, P. Buckley. Binding of Pu to galacturonic acid and extracellular polymeric substances (EPS) from *Shewanella putrefaciens*, *Clostridium* sp and *S. putrefaciens*. [Manuscript in preparation].
- Kantar, C. and B.D. Honeyman. Binding of Pu to citric and alginic acids. [Manuscript in preparation].
- Roberts, K., Santschi, P.H., Leppard, G.G., and West, M. 2004a. Composition of colloids from surface waters and ground waters in Colorado. *Colloids and Surfaces*, accepted.
- Roberts, K., and Santschi, P.H. (2004). Inverse dependency of particle residence times in ponds to the concentration of phosphate, the limiting nutrient. *J. Environ. Radioactivity*, in press.
- Roberts, K.A., Santschi, P.H., and Honeyman, B.D. 2004b. Exopolymeric Substances (EPS) acting as a colloid trap in the ternary system Plutonium – EPS - colloidal silica. [Manuscript in preparation.]
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