MICROBIAL REDUCTION OF URANIUM

Y. SUZUKI[1] & J. F. BANFIELD[2]

[1]Subground Animalcule Retrieval (SUGAR) Project, Frontier Research System for Extremophiles
Japan Marine Science & Technology Center (JAMSTEC), Yokosuka, Japan
[2]Department of Earth and Planetary Sciences, University of California Berkeley, Berkeley, USA

SUMMARY – Fate of U(IV) solid phases under oxidizing conditions is of great environmental importance. In shallow aquatic sediment, U(VI) was reduced to U(IV) directly by bacteria including *Geobacter* and *Desulfovibrio* spp., but not by the aqueous sulfide or iron sulfide minerals produced by sulfate-reducing bacteria. In shallow subsurface sediment nearby the aquatic sediment, uranium was as enriched as economically important deposits and the majority of uranium persisted as U(IV) against reoxidation. The shallow subsurface sediment was colonized by a microbial community actively cycling nitrogen, iron and sulfur. Preservation of U(IV) in oxidizing sediments is attributed to rapid microbial utilization of oxygen, nitrate and Fe(III), which rapidly oxidize U(IV).

1. INTRODUCTION

The redox transformations between insoluble U(IV) and soluble U(VI) have controlled uranium mobility throughout Earth's history (Maynard 1983, Dahlkamp 1993, Klinkhammer and Palmer 1991). The reduction of U(VI) to U(IV) that precipitates in minerals occurs under reducing conditions. The oxidative dissolution of U(IV) minerals is rapid when oxidizing agents such as oxygen, nitrate and Fe(III) are available (Nevin et al. **2000).** The reduction of U(VI) in organic-rich terrestrial sediments led to formation of economically important uranium ore deposits (Maynard 1983, Dahlkamp 1993). The reductive precipitation of U(IV) minerals in organic-rich marine sediments is inferred to be the most significant modem global sink for dissolved uranium (Klinkhammer and Palmer 1991, Barnes and Cochran 1993). Despite of the importance of U(VI) reduction, the key pathways for U(VI) reduction in natural organic-rich sediments remain controversial.

The discovery that microorganisms can reduce U(VI) and precipitate U(IV) minerals in the laboratory (Lovley et al. 1991) spurred the development of the cost-effective in-situ bioremediation technology to immobilize uranium using microbial reduction of U(VI) (Lovley 2001). Spent nuclear fuels containing more than 80% U(IV) as a solid phase, UO_2, will be disposed of into the oxidizing subsurface strata (Wronkiewics and Buck 1999). However, the resilience of U(IV)-bearing solid **phases** under oxidizing conditions with microbial activity remain unclear.

2. MATERIAL AND METHODS

2.1 Sample collection

The aquatic and subsurface sediments were collected fiom the open pit #4 (pit 4) at the Midnite mine in Washington State, USA. The sediments were kept cold during shipment.

2.2 Geochemical characterizations

The pH of surface and pore waters were measured on site. Fe(II), Fe(total), Mn, nitrate, sulfate, and sulfide were measured by spectrophotometer on site for the surface water and in the laboratory for pore waters (Hach). Pore waters were extracted anaerobically by centrifugation in the laboratory within three days after the sample collection. Alkalinity was measured by colorimetric titration on site (Hach). Total organic carbon was determined using a high-temperature combustion carbon analyzer (Tekmar Dohrmann). Elemental concentrations of uranium, copper, and calcium were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (Jarrell). All samples were filtrated with **0.2** □m-pore membrane filters for analyses.

2.3 Measurements for elemental concentrations in the sediments

Aquatic sediments were centrifuged, and supernatants (pore waters) were removed under anaerobic conditions. The pellets of the aquatic sediment and the sediment fiom shallow subsurface were freeze-dried. Whole dried sediments were digested with $HNO_3/HClO_4$ (6:1), followed by with ICP-OES analyses

2.4 X-ray absorption near-edge structure (XANES) measurements

XANES measurements were made under anaerobic conditions on the wet homogeneous sediments held in a plexiglass sample holder and sealed with Kapton film. Sediment XANES data were compared to the U(IV) and U(VI) standards (See supplementary method for further details).

2.5 Scanning and transmission electron microscopes

The aquatic sediment was freeze-dried and embedded into epoxy resin after removal of the pore water by anaerobic centrifugation. Polished surface of the solidified epoxy resin was coated with carbon and characterized using a LEO 1530 scanning electron microscope. The aquatic sediment was mounted on Ni grids coated with formvar films, and characterized using a Philips CM200UT transmission electron microscope.

2.6 16S rRNA gene sequence analysis

Genomic DNA in the aquatic and subsurface sediments were extracted using the ultraclean. soil DNA kit (Mo Bio). 16S rRNA gene sequences were amplified, cloned and sequenced as previously described (Bond et al. 2000). A bacteria-specific primer set (27F and 1492R) was used for PCR amplification. Distance analysis was performed using unambiguously aligned 575 homologous bases.

2.7 MPN-PCR

PCR-MPN was carried out with nested PCR with the first round of a bacteria-specific primer set (27F and 1492R) and the second round of a *Desulfovibrio*-specific primer set (230F and 838R) (Daly et al. 2000) approximately following a method previously described (Picard et al. 1992). DNA extraction and PCR amplification were done in triplicate.

3. RESULTS AND DISCUSSION

3.1 Geochemical characterizations of the pit 4 sediments

Naturally organic-rich sediments occur in the pit 4 at the abandoned Midnite uranium mine in Washington, USA. By the time mining ceased in 1981, most of the uranium ore in the vicinity of P4 had been removed. The water is dilute, slightly alkaline, and has maintained a uranium concentration of ~ 2-4 ppm, only slightly higher than the average U content of the crust (Taylor 1964). Abundant organic matter (~1% in sediments and ~3 ppm dissolved organic carbon, DOC, in surface water) is generated by algae and surrounding vegetation. The site is hot and dry in summer, but abundant water is supplied in the form of snow each winter. This leads to fluctuation in the pond water level and periodic exposure of the sediments to air.

In June of 2001, black sediment from ~2 cm below the water surface (see supplemental figure 1), surface water, and pore water were analyzed (Table 1). Surface water was oxygenated, slightly alkaline, and contained 2.67 ppm U. However, pore water was neutral pH and enriched with uranium (~ 8 ppm) and organic matter (787 ppm DOC). Fe(II) and sulfide were also enriched, suggesting coupling of oxidation of organic matter to Fe(III) and sulfate reduction.

Table 1. Geochemical characteristics of [1] surface P4 water, [2] in-situ pore water in aquatic sediment, and [3] the pore water incubated for 30 days under anoxic conditions (mg/L, otherwise indicated). All values are mean from at least duplicate measurements and range within less than 10% errors, otherwise indicated.

	[1]	[2]	[3]
Temp. (°C)	19.4	19.6	23.5
pH (unit)	7.82	7.01	6.74
DOC	3.21	787.24	5366.42
Alkalinity	42.4	ND*	ND
Fe(II)	0.01	9.44	10.72
Fe(total)	0.07	9.48	10.87
Mn	0.77	18.22	5.91
NO_3^-	3.14	0.82	0.63
SO_4^{2-}	354.20	246.25	123.12
S^{2-}	<0.001	0.06	0.16
Ca	138.01	198.88	221.96
Cu	0.135	0.17	0.16
U	2.67	7.30	0.22

*Not determined in this study.

3.2 Mineralogical characterizations of the pit 4 sediments

The aquatic black sediment contained ~ 500 ppm U, analytically determined after digestion with $HNO_3/HClO_4$. X-ray absorption near-edge structure (XANES) analysis of this sediment revealed that 80±10% of the uranium was present as U(IV), in contrast to previous studies showing organic-rich sulfidogenic sediment containing only 25% U(IV) (Duff et al. 1997). Examination of the sediment by scanning electron microscopy (SEM) with energy dispersive x-ray (EDX) microanalysis showed that uranium was co-located with iron and sulfur (Figure 1A). Framboidal pyrite (FeS_2; Fig. 1B) and algal detritus were abundant.

Transmission electron microscope (TEM)-based EDX analyses showed uranium was not strongly associated with pyrite or algal cells, although it

has previously been suggested that these sequester uranium through adsorption (Wersin et ak. 1994, Degens et al. 1977). Some prokaryotic cells were coated with amorphous –2 nm FeS particles (Fig. 1C), indicating presence of sulfate-reducing bacteria (SRB). Other morphologically distinct prokaryotic cells were enriched in uranium, iron, and sulfur (Fig. 1D). A subset of cells coated by highly reactive nanometer-scale FeS particles did not concentrate uranium. This suggests that uraninite formation is not simply due to inorganic reduction by sulfide ions or sulfide mineral surfaces (Fig. 1C). These data also suggest that only a subset of the SRB in the sediment are capable of U(VI) reduction.

After one-month incubation of this sediment under N_2 without any amendment, the uranium pore water concentration decreased to 0.22 ppm (Table 1). More sulfide was produced, as indicated by decrease in sulfate and increase in sulfide in the pore water (Table 1). The sediment uranium content increased to 750 ppm. XANES analysis for this incubated sediment indicated that 70± 10% of the uranium was in the form of U(IV). These results strengthen the conclusions that reduced uranium in the sediment is due to microbial activity and is not simply in the form of detrital particles.

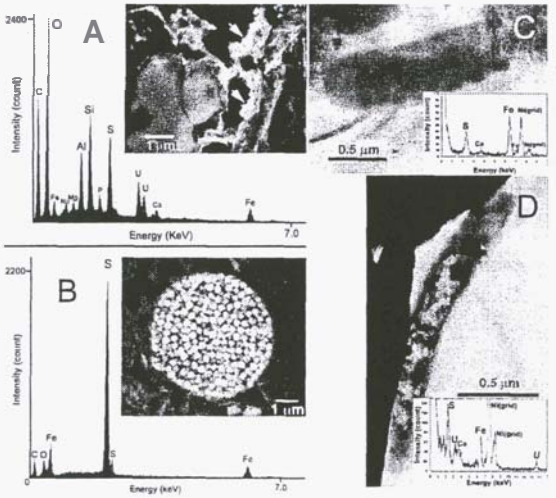

Figure 1. (A) Backscattered-electron image (BEI) of uranium-bearing phases (see arrows) found in the aquatic sediment, and associated EDX spectrum. (B) BEI of framboidal pyrite in the sediment, and associated EDX spectrum. (C) TEM image and EDX spectrum (–30 nm spatial resolution) from of a prokayotic cell in the sediment that has accumulated iron and sulfur. (D) TEM image and EDX spectrum of a prokaryotic cell from the sediment that has accumulated uranium, iron and sulfur. The U concentration is approximately 10^4 x higher than that of associated pore water solutions, implying significant localization of U due to microbial activity.

3.3 Microbial characterizations of the pit 4 sediments.

DNA were extracted fiom the shallow aquatic sediment and 16S rRNA genes were amplified by PCR with a primer set specific to the genus *Desulfovibrio,* all members of which have been shown in the laboratory to reduce sulfate and U(VI) (Lovley and Phillips 1992, Lovley et al. 1993). PCR accompanied by most probable number method (PCR-MPN) was conducted to enumerate the *Desulfovibrio* cell. The result showed that $2.8\pm0.4 \times 10^5$ (n=3) *Desulfovibrio* cells per one gram of sediment without the pore water were detected in the sediment. Thus, the presence of sulfate-reducing bacteria capable of U(VI) reduction was also confirmed by 16S rRNA gene analysis.

Due to the fluctuation of the water level of P4, the sediment that was wet and black in June of 2000 was dry and yellowish brown in June of 2001. In order to investigate the fate of biologically reduced U(IV) against reoxidation, we sampled shallow subsurface sediment 10 cm below the sediment-air interface (see supplemental figure 2). The subsurface sediment was black and contained 780 ppm U (table 1), indicating that U was as enriched as some economically important U ore deposits. Although the sediments below and above the black layer were oxidized as indicated by the colors, 75±10% of uranium enriched in the subsurface black sediment remained in the U(IV) form, which is different fiom the other surficial uranium ore deposits that were enriched exclusively with U(VI).

DNA was also extracted from the shallow subsurface black sediment and 16S rRNA genes were amplified with a bacteria-specific primer sets and cloned. Analysis of the DNA sequences obtained suggested the sediment was colonized by a microbial community with high phylogenetic diversity (Fig. 2). Of 52 clones analyzed, eight clones were closely related to microaerophilic sulfur-oxidizing and nitrate-reducing *Thiobacillus thioparus* (Kelly and Wood 2000). Organisms closely related to a microaerophilic Fe(II)-oxidizing and O_2-reducing strain (Emerson and Moyer 1997) and microaerophilic ammonia-oxidizing and O_2-reducing *Nitrosospira multiformis* (Madigan et al. 1997) were abundant. From the results, it was indicated that oxygen and nitrate, which rapidly oxidize U(IV), were reduced by the microaerophilic bacteria. In addition to the microaerophilic bacteria, organisms related to anaerobic Fe(III)- and U(VI)-reducing *Geobacter metallireducens* (Lovley et al. 1991), anaerobic sulfate-reducing *Desulfosarcina variabilis* and *Fusibacter paucivorans,* and anaerobic N_2-fixing phototrophic *Rhodopseuodomonas* sp. were present. It is also indicated based on this DNA analysis that nitrogen, iron and sulfur were being cycled among

the microaerophilic and anaerobic bacteria. As Fe(III) is also known to rapidly oxidize U(IV), microbial reduction of Fe(III) may have contributed to preservation of U(IV). Thus, U(IV) persisted under oxidizing conditions owing to microbial nitrogen, sulfur and iron cycling.

These results provide the first direct insights into the detailed geomicrobiology of natural uranium cycling in near-surface sediments and clearly indicate the importance of microorganisms in determining the fate of uranium in the environment. Microbial reduction of U(VI) can be stimulated by amendment with organic matters[19] and have great potential to provide an inexpensive and feasible way to rapidly recover uranium from solution for mining and environmental remediation.

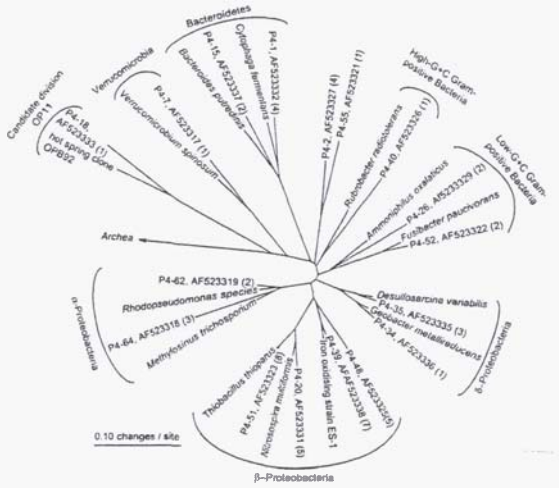

Figure 2. Phylogenetic analysis of 16S rRNA gene sequences obtained from the subsurface black sediment and from GenBank. The tree was constructed by Jukes-Cantor distance analysis. The number of clonal sequences with >98% similarity to the representative sequence is shown in parenthesis. *An Archaeon, Pyrobaculum ishlandis,* was used as the outgroup.

4. ACKNOWLEDGEMENTS

The authors thank D. Bruce and E. Hale for assistance with sample collections. YS thanks Yoshida Scholarship for financial support.

5. REFERENCES

Bond, P. L., Smriga, S. P. and Banfield, J. F. (2000) Phylogeny of microorganisms populating a thick, subaerial, predominantly lithotrophic biofilm at an extreme acid mine drainage site. *Appl. Env. Microbiol.* 66, 3842-3849.

Branes, C. E. and Cochran, J. K. **(1993)** Uranium geochemistry in estuarine sediments: controls on removal and release processes. *Geochim. Cosmochim. Acta. 57,* 555-569.

Dahlkamp, F. J. in Uranium ore deposits (Springer-Verlag, Berlin, **1993).**

Daly, K., Sharp, R. J. and McCarthy, A. J. **(2000)** Development of oligonucleotide probes and PCR primers for detecting phylogenetic subgroups of sulfate-reducing bacteria. *Microbiol.* **146: 1693-1705.**

Duff, M. C., Amrhein, C., Bertsch, P. M. and Hunter, D. B. **(1997)** The chemistry of uranium in evaporation pond sediment in the San Joaquin Valley, California, USA, using X-ray fluorescence and XANES techniques. *Geochim. Cosmochim. Acta.* **61, 73-81.**

Emerson, D. and Moyer, C. **(1997)** Isolation and characterization of novel iron-oxidizing bacteria that grow at circumneutral pH. *Appl. Environ. Microbiol.* 63, 4784-4792.

Kelly, D. P. & A. P. Wood. **(2000)** Confirmation of *Thiobacillus denitrificans* as a species of the genus *Thiobacillus,* in the b-subclass of the *Proteobacteria,* with strain NCIMB **9548** as the type strain. *Int. J. Syst. Evol. Microbiol. 50,* 511-516.

Klinkhammer, G. P. & Palmer, M. R. **(1991)** Uranium geochemistry. *Geochim. Cosmochim. Acta.* **55, 1799.**

Lovley, D. R. E. **(2001)** Anaerobes to the rescue. *Science* **294, 1444-1446.**

Lovley, D. R., Phillips, E. J. P., Gorby, Y. A. and Landa, E. R. **(1991)** Microbial reduction of uranium. *Nature* **350,413-416.**

Lovley, D. R. and Phillips, E. J. P. **(1992)** Reduction of uranium by *Desulfovibrio desulfuricans. Appl. Environ. Microbiol.* **58, 850-856.**

Lovley, D. R., Roden, E. E., Phillips, E. J. P. and Woodward, J. C. **(1993)** Enzymatic iron and uranium reduction by sulfate-reducing bacteria. *Marine Geol.* 113, 41-53.

Madigan, M. T., Martinko, J.M. & Parker, J. in biology of microorganisms (Prentice Hall, New Jersey, **1997).**

Maynard, J. B. in Geochemistry of sedimentary ore deposits (Springer-Verlag, New York, **1983).**

Nevin, K. P. and Lovley, D. R. (2000) Potential for nonenzymatic reduction of Fe(III) during microbial oxidation of organic matter coupled to Fe(III) reduction. *Environ. Sci. Technol.* **34,2472-2478.**

Picard, *C.*, Ponsonnet, C., Paget, E., Nesme, X. and Simonet, P. **(1992)** Detection and enumeration of bacteria in soil by direct **DNA** extraction and polymerase chain reaction. ***Appl. Env. Microbiol.* 58,2717-2722.**

Taylor, **S.** R. **(1964)** Abundance of chemical elements in the continental crust: a new table. ***Geochim. Cosmochim. Acta.* 28, 1273-1284.**

Wersin, **P.,** Hochella, M. F. Jr, Persson, P., Redden, R., Leckie, J. **O. and** Harris, D. **(1994)** Interaction between aqueous uranium(VI) and sulphide minerals: spectroscopic evidence for sorption **and** reduction. ***Geochim. Cosmochim. Acta.* 58,2829-2843.**

Wronkiewics, D. J. and Buck, E. C. **(1999).** Uranium mineralogy and the geologic disposal of spent nuclear fuel. in ***Uranium: Mineralogy, Geochemistry and the Environment*** (eds Burns, P. C. & Finch, R.) *Rev.* ***in Mineral.,38,* 475-497**