Uranium Reduction by *Clostridia*

A.J. Francis, Cleveland J. Dodge, and Jeffrey B. Gillow
Brookhaven National Laboratory

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Outline

- Growth of *Clostridium* sp. under normal culture conditions.
- Fate of metals and radionuclides in the presence of *Clostridia*.
- Bioreduction of uranium associated with nitrate, citrate, and lepidocrocite.
- Utilization of *Clostridium* sp. for immobilization of uranium at the FRC Area 3 site.

Background

- The FRC groundwater and sediment contain significant concentrations of U and Tc and are dominated by low pH, and high nitrate and Al concentrations where dissimilatory metal reducing bacterial activity may be limited.
- The presence of *Clostridia* in Area 3 at the FRC site has been confirmed and their ability to reduce uranium under site conditions will be determined.
- Although the phenomenon of uranium reduction by *Clostridia* has been firmly established, the molecular mechanisms underlying such a reaction are not very clear.

  - we are exploring the hypothesis that U(VI) reduction occurs through hydrogenases and other enzymes (Matin and Francis).
- Fundamental knowledge of metal reduction using *Clostridia* will allow us to exploit naturally occurring processes to attenuate radionuclide and metal contaminants in situ in the subsurface.

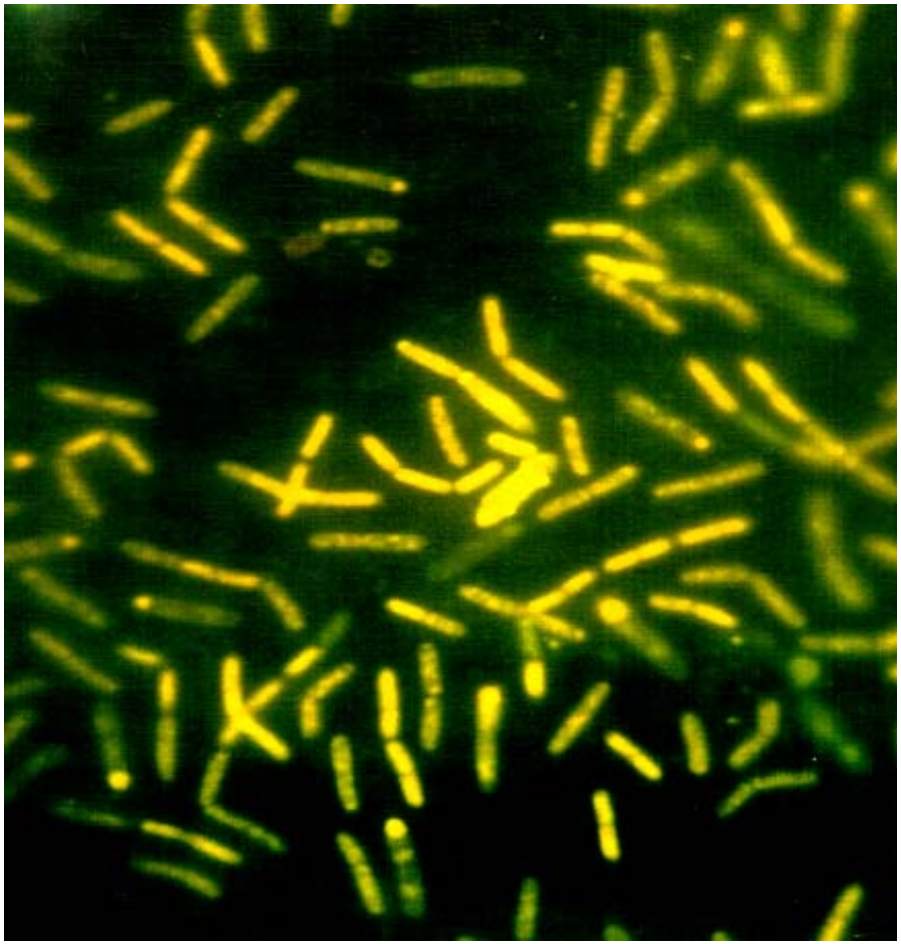
Materials and Methods

Bacterial culture: Obligate anaerobic bacteria *Clostridium* sp. (ATCC 53464) capable of utilizing glucose, and a citrate degrading bacterium *Clostridium sphenoides* (ATCC 19403).

Bioreduction studies: Growth medium (pH 6.8) containing glucose and inoculated with *Clostridia* and U-nitrate, U-citrate or Fe-U-coprecipitate added at the start of the experiment.

Metal speciation: *Uranium*: oxidation state determined in solution by KPA and UV-vis spectrophotometry, in solid phase by XPS and XANES, molecular association by EXAFS.
Technetium: gel filtration chromatography, gamma-counting.
Plutonium: liquid scintillation counting, XANES.

Clostridium sp.



Cell size: 1 μm x 5 μm

- Strict anaerobic, spore-forming, fermentative bacteria.
- Metal reduction

  Fe^{3+} to Fe^{2+}

  Mn^{4+} to Mn^{2+}

  Tc^{7+} to Tc^{4+}

  U^{6+} to U^{4+}
- Mobilization/Immobilization

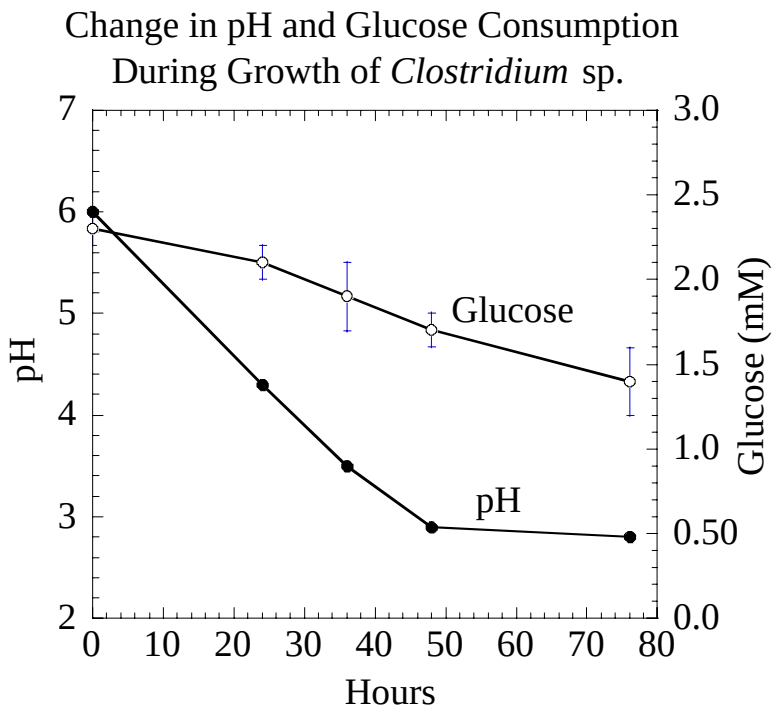
  - U(VI)-nitrate$_{(aq)}$ → U(IV)s

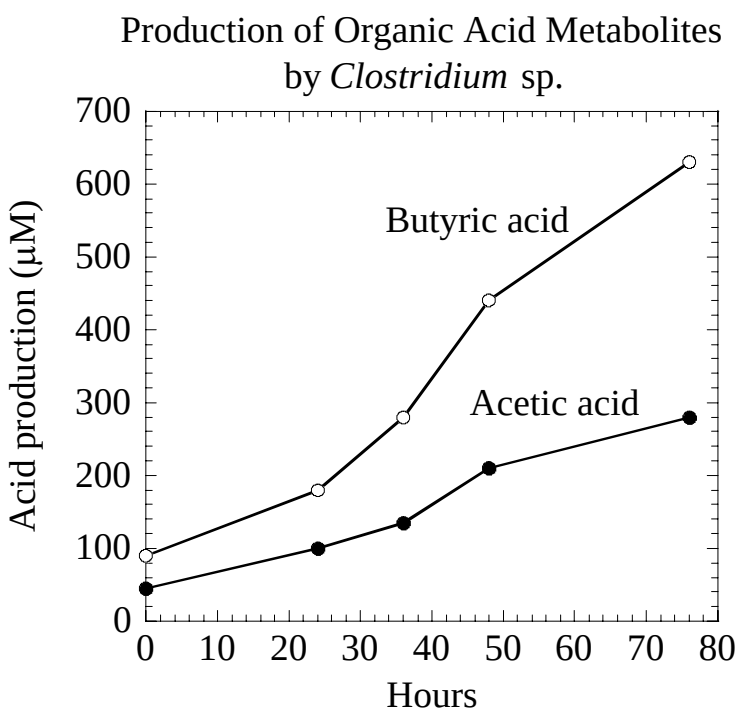
  - U(VI)-citrate$_{(aq)}$ → U(IV)-citrate$_{(aq)}$

Serum Bottles for Culturing Anaerobic Bacteria

Anaerobic growth medium is prereduced and autoclaved. The metal-containing solution is added through the butyl rubber stopper using a needle and syringe.

Growth of *Clostridium* sp.



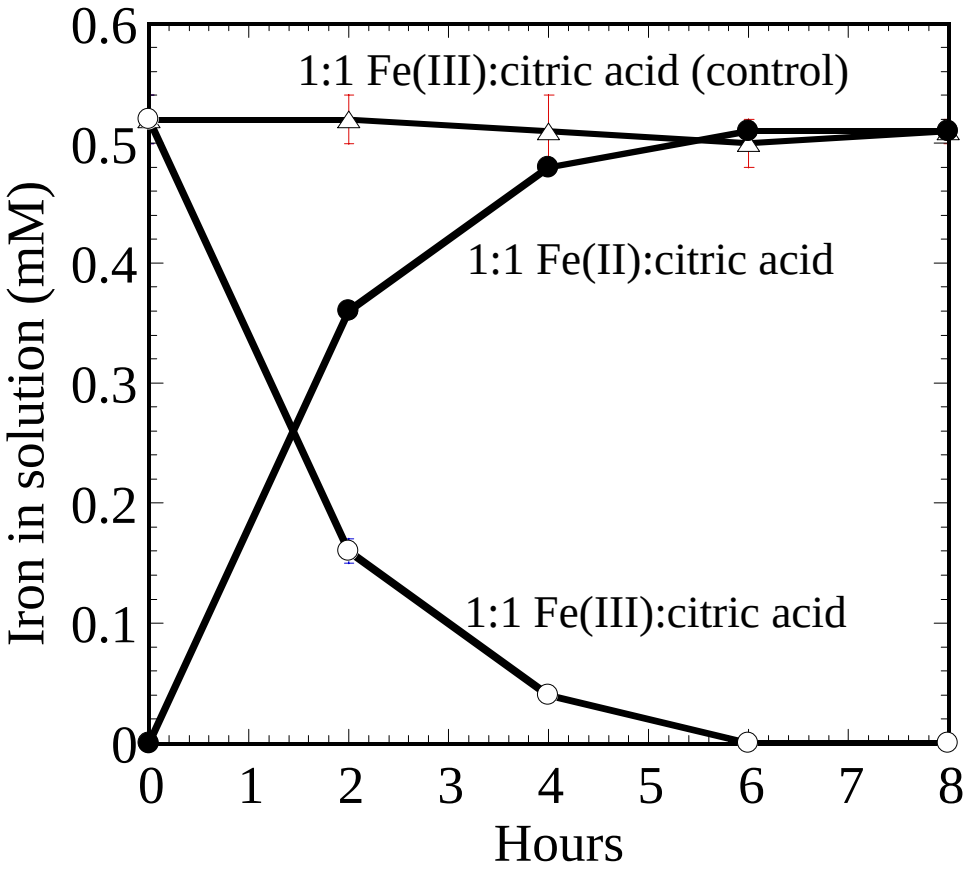


Clostridium sp. rapidly metabolizes glucose to acetic and butyric acids which lowers the pH of the medium. Equimolar amounts of carbon dioxide and hydrogen are also produced.

Toxicity effects (e.g. Pb) can be minimized by addition of iron. (Francis and Dodge. 1987. Arch. Env. Contam. Toxicol. 16:491-498).

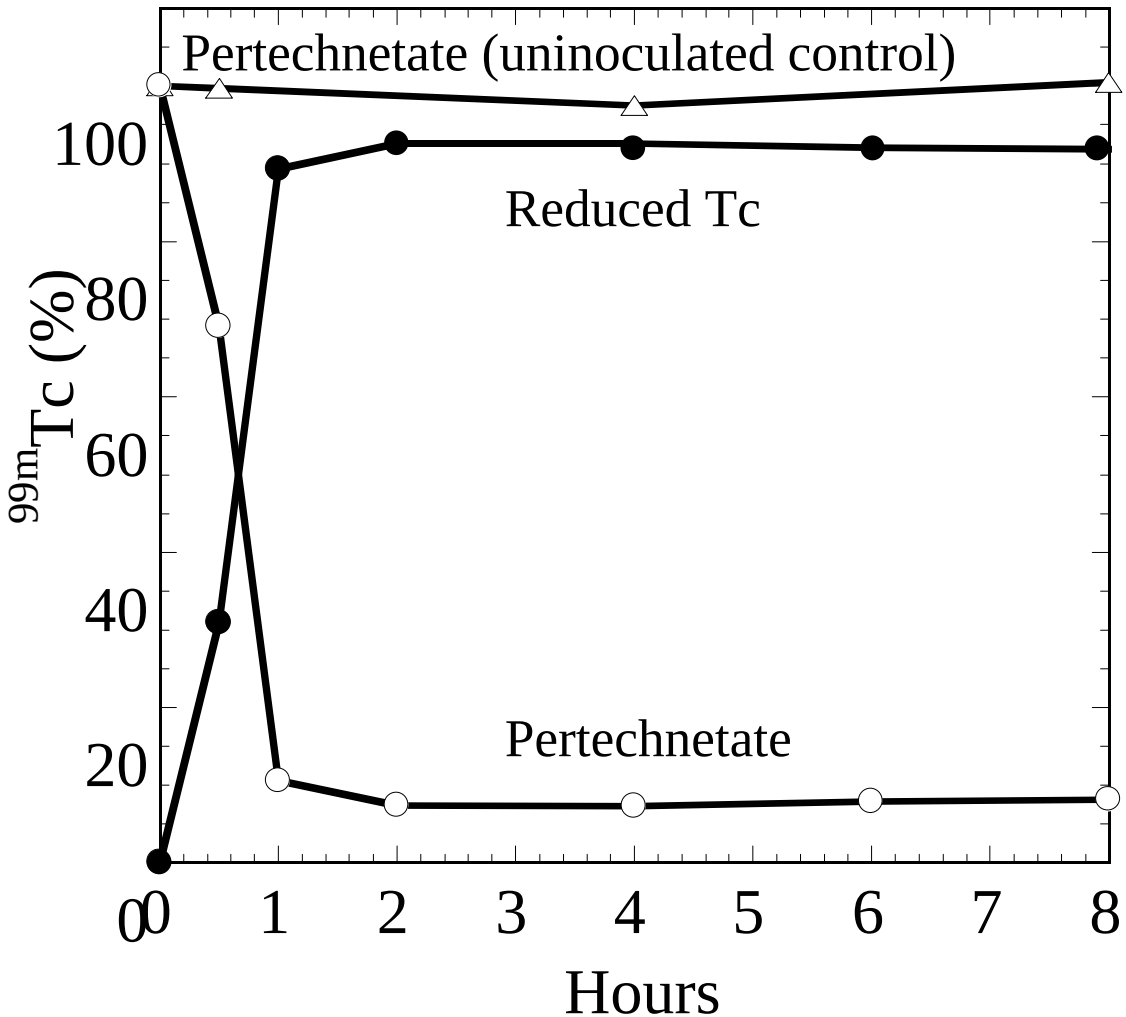
Metal Reduction by *Clostridium sp.*

Bioreduction of 1:1 Fe(III)-citrate complex by *Clostridium* sp.



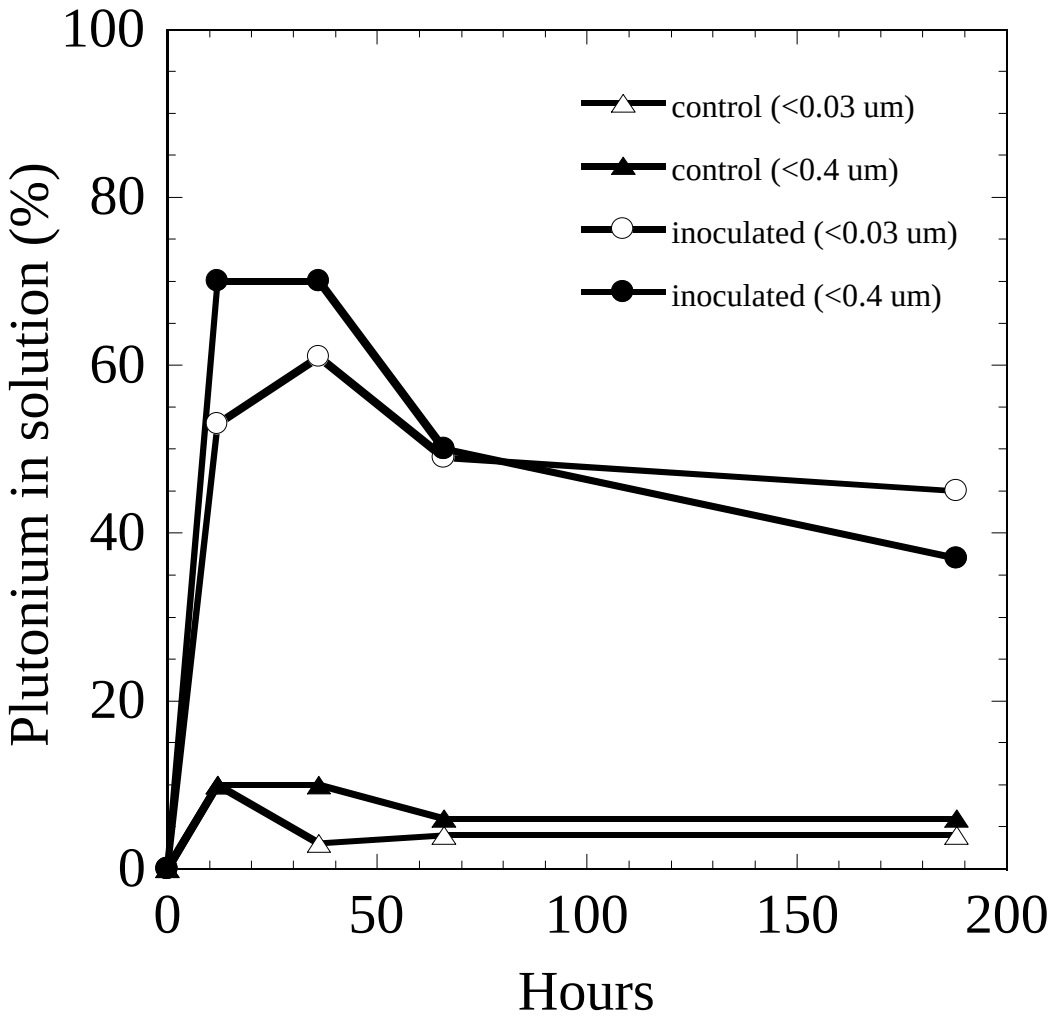
Ferric ion was reduced to ferrous form by the bacteria and remained in solution as the 1:1 Fe(II):citric acid complex. (Francis and Dodge. 1993. Appl. Env. Microbiol. 59:109-114).

Bioreduction of Pertechnetate by *Clostridium sp.*



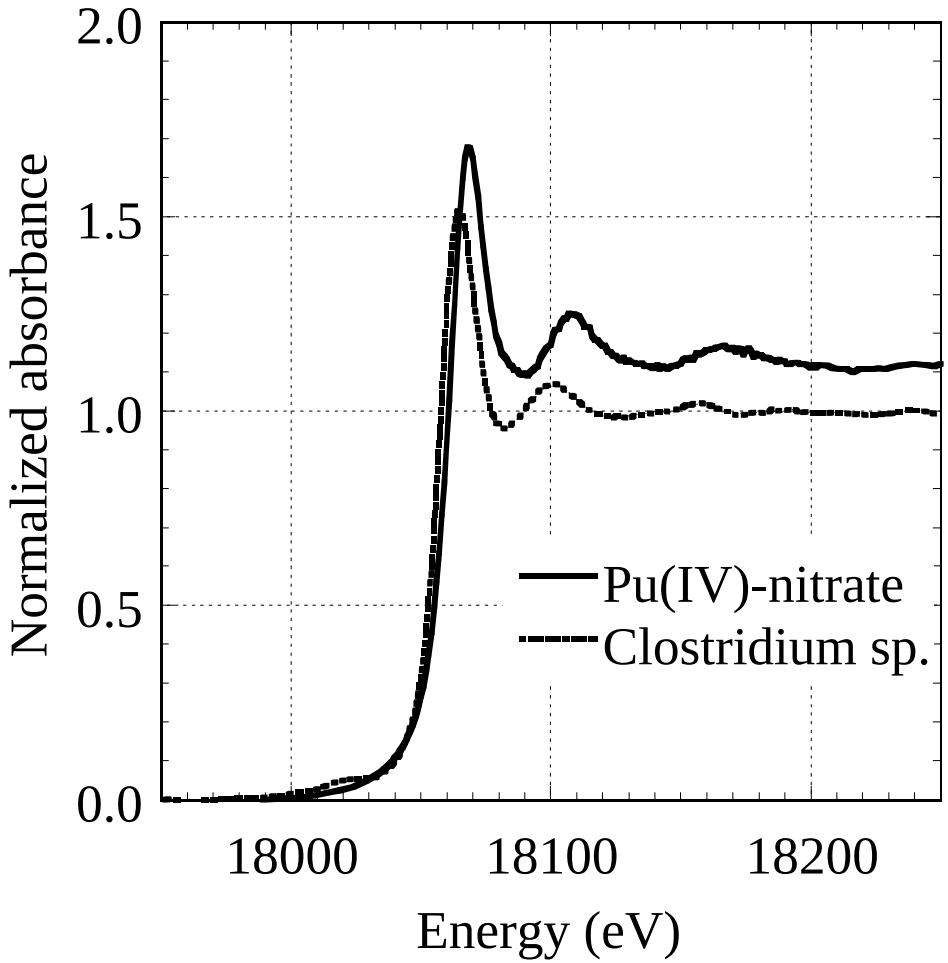
Pertechnetate ($NaTcO_4$) added to resting cells of *Clostridium* sp. was rapidly reduced to Tc(IV) by bacterial activity. The reduced Tc was associated with the biomass and in solution complexed with >5kDa metabolites. Tc(VII) added in the presence of citrate or DTPA formed soluble Tc(IV)-organic complexes (Francis et al. 2002. Radiochim. Acta 90:791-797).

Dissolution of Pu Species by the Activity of *Clostridium* sp.



Pu(IV)-nitrate was added to the growth medium and initially precipitated from solution. Anaerobic bacterial activity solubilized the Pu.

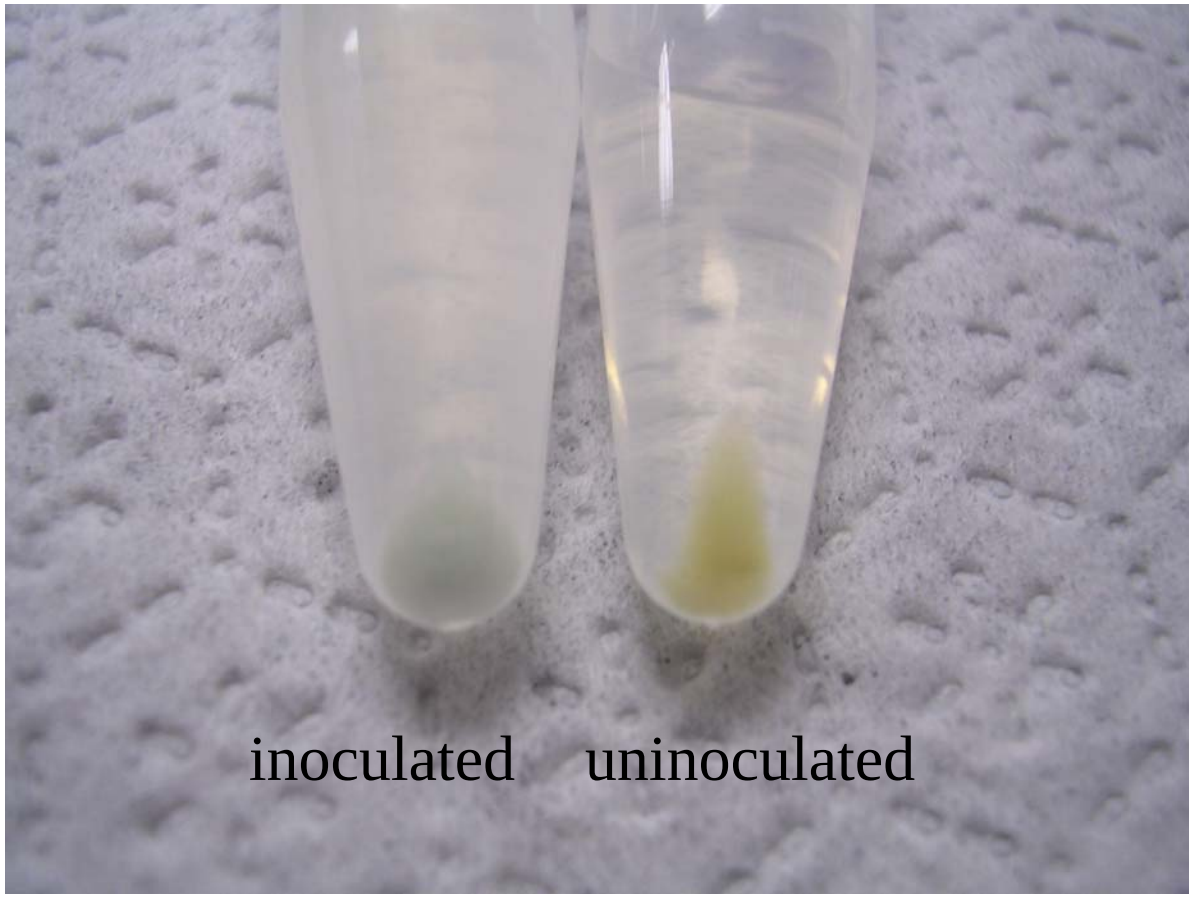
XANES Analysis of Pu Following Anaerobic Bacterial Activity



Comparison of the absorption edge position for the bacterially treated sample at (18.059 keV) with that for Pu(IV)-nitrate (18.062 keV) confirms the presence of Pu^{3+}.

Uranium Reduction by *Clostridia*

Uranium Speciation in the Presence of *Clostridium* sp.



The uranyl ion has a characteristic yellow color (L), while the bacterially-treated uranium (R) has a dark color.

XPS and XANES Analysis of Uranium Following Anaerobic Bacterial Activity

XPS analysis of the uranyl nitrate treated sample shows a 1.6 eV decrease in binding energy to 380.6 eV compared to uranyl ion (382.0 eV); XANES spectra at the M_V absorption edge shows shift in sample absorption peak to 3550.1 eV from 3551.1 eV for U(VI). These complementary techniques confirm bacterial reduction of uranyl ion to U(IV). Francis et al. 1994. Environ. Sci. Technol. 28:636-639.

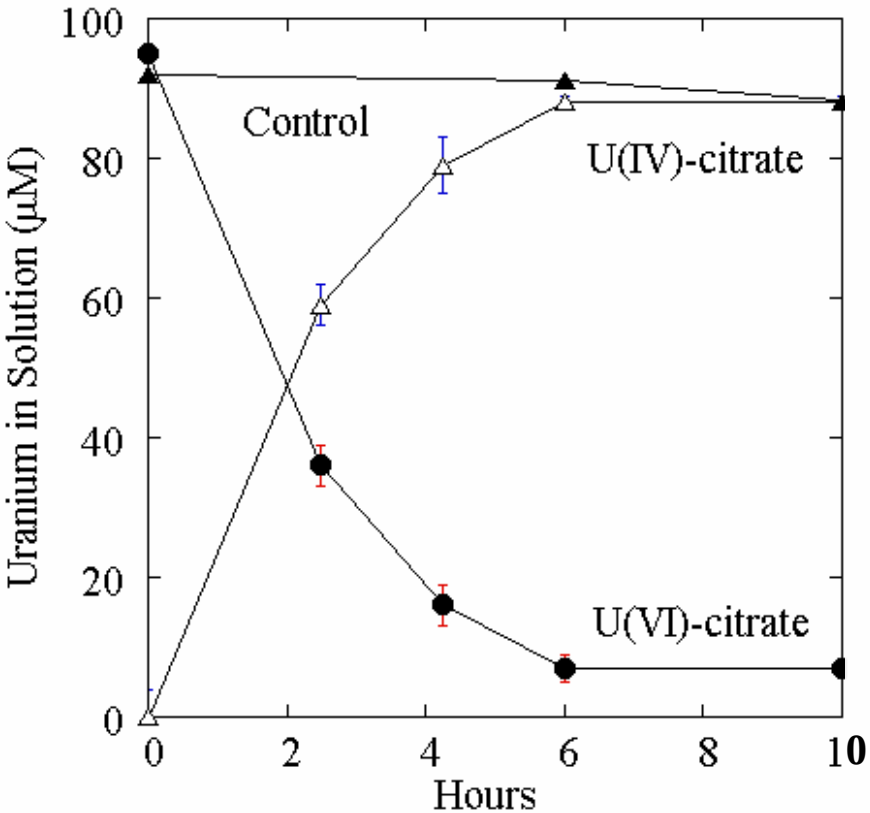
Mechanisms of Uranium Reduction by *Clostridium* sp.

Treatment	U^{4+} detected (%)
Control (no bacteria)	none detected
Synthetic spent medium (org. acids)	none detected
Cell free spent medium (filtered)	1
Cell free spent medium (filtered/autoclaved)	none detected
Heat-killed cells	2
Resting cells (no growth)	94
Growing cells	100

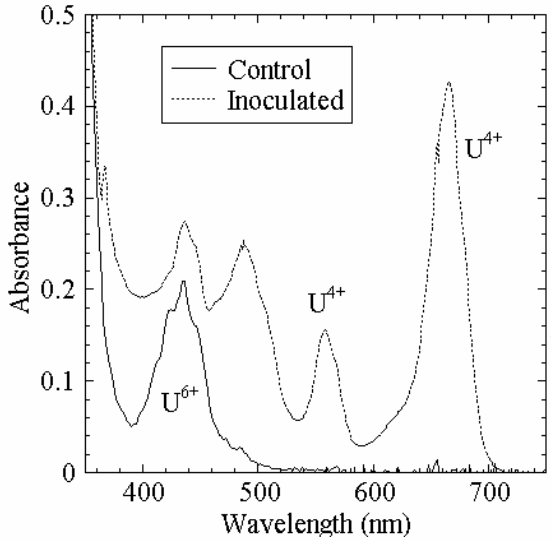
210 μM as uranyl acetate was added.
Samples were analyzed at 24 -h following addition of uranium.

Reduction of uranyl nitrate was observed only in the presence of cells. The components of the growth medium and the metabolic acids did not reduce uranium.

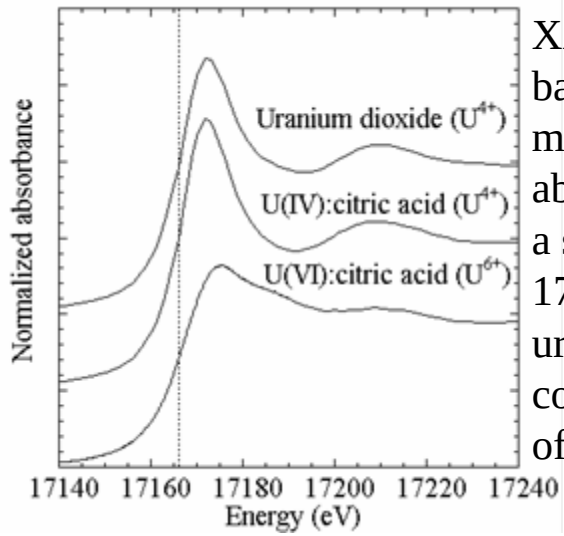
Anaerobic Bacterial Reduction of Uranium Complexed With Citric Acid



Clostridium sp. reduced U(VI) complexed to citric acid only in the presence of carbon source. The reduced U remained in solution associated with the citric acid as the U(IV)-citrate complex.



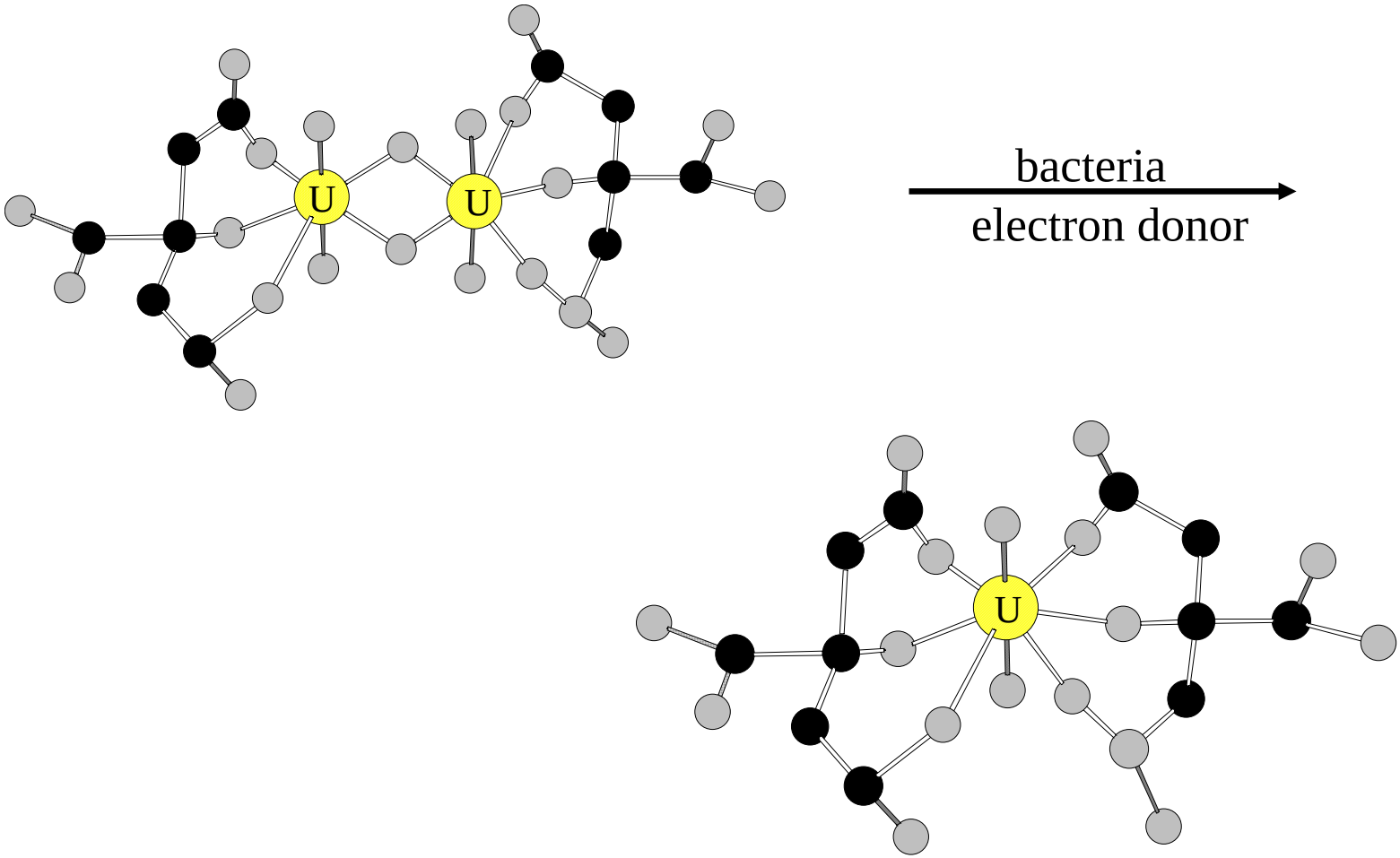
Change in UV-vis spectrum of U(VI)-citrate following bioreduction confirms it was reduced to U(IV).



XANES analysis of the bacterial growth medium at the L_{III} absorption edge shows a shift in energy to 17166 eV compared to uranyl ion (17171 eV), confirming reduction of U(VI) to U(IV).

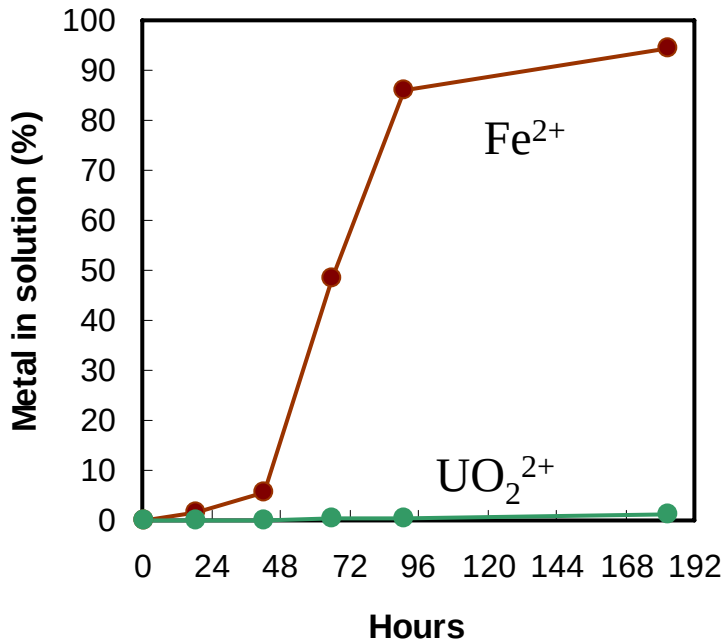
Francis et al., 2002. J. Nucl. Sci. Technol. 3:935-938.

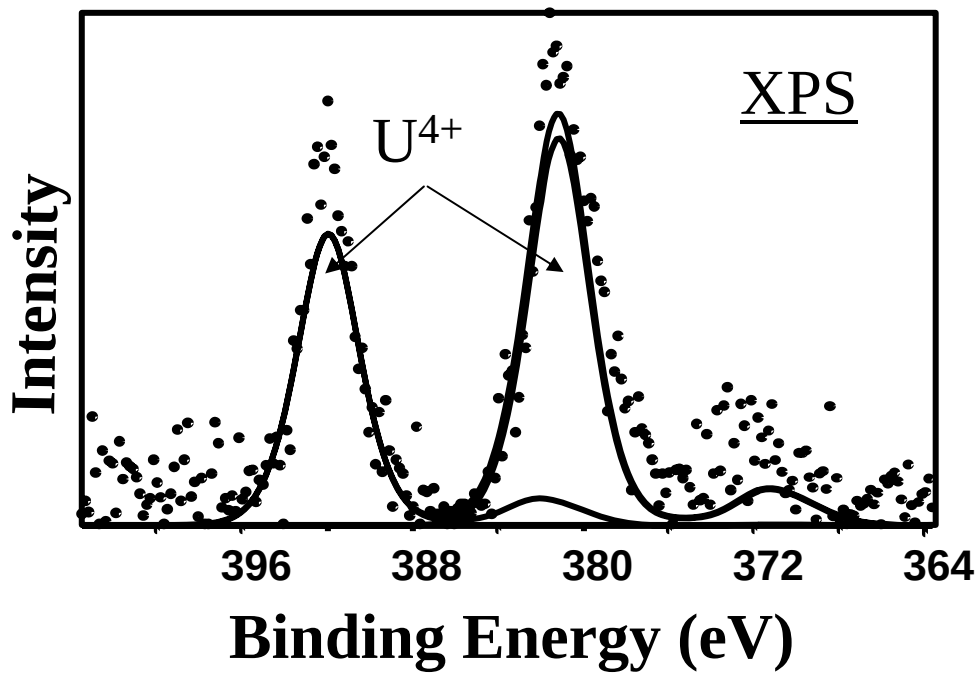
Proposed Structure for U(IV)-Citrate Complex



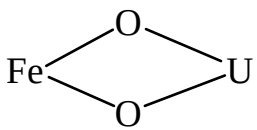
EXAFS analysis indicates the binuclear U(VI)-citrate complex is transformed to a mononuclear biligand complex following reduction of U(VI) to U(IV).

Bioreduction of Uranum-Lepidocrocite Coprecipitate by *Clostridium* sp.





EXAFS analysis has shown uranium forms an inner-sphere complex with lepidocrocite .



Anaerobic bacterial activity solubilized the iron as ferrous form; however, uranium was not solubilized due to formation of U^{4+}. (Dodge et al. 2002. Environ. Sci. Technol. 36:3504-3511).

Uranium Reduction by *Clostridium* sp. in Groundwater Collected at the FRC

Field Research Center

Methodology

Groundwater: Groundwater samples FW-024-000233 and FW-026-000343 from Area 3 of FRC site were obtained from Dave Watson and were characterized using potentiometric titration, ICP-OES, and EXAFS.

Microbial experiments:

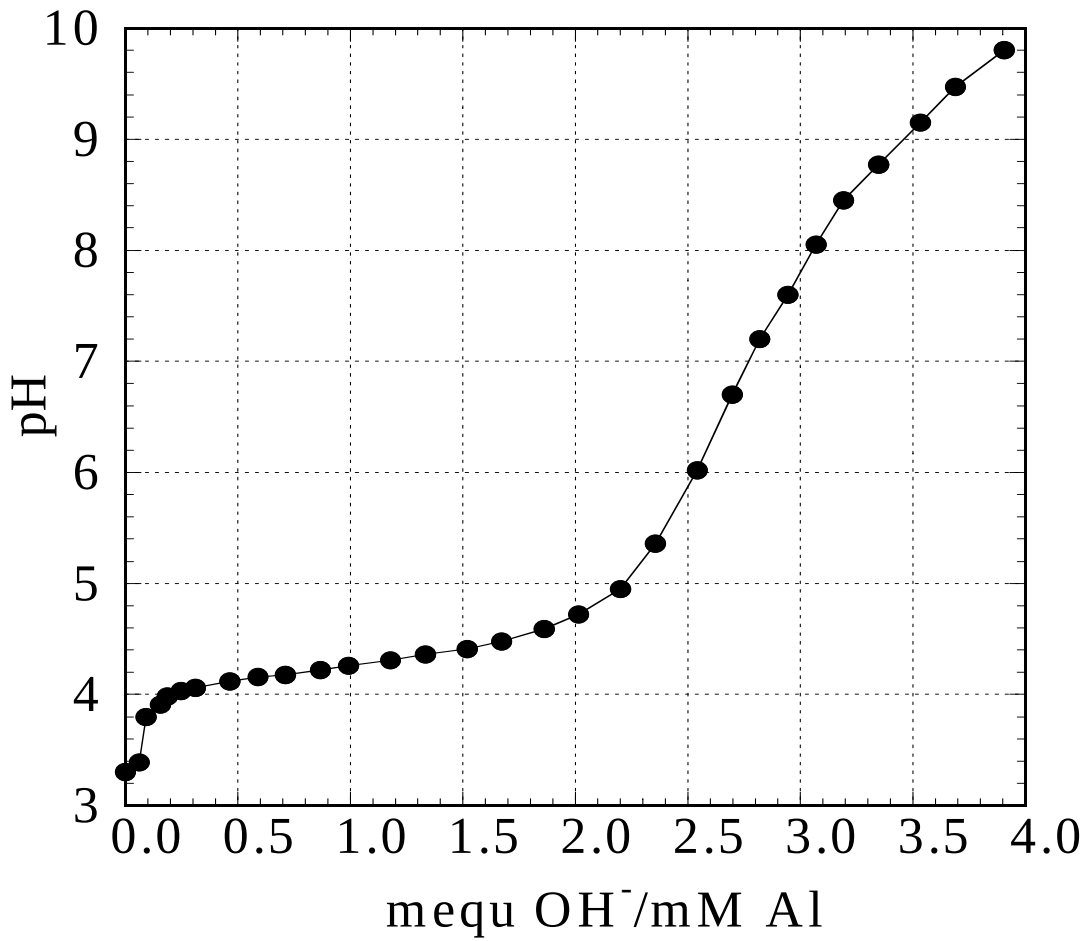
- The ability of indigenous bacteria to reduce U was tested on "as received" and pH 6.5 adjusted groundwater.
- Uranium reduction by log-phase *Clostridium* sp. was determined in the presence of groundwater, uranium, and aluminum.
- Growth was monitored by measuring optical density (600 nm), pH, and glucose consumption.
- Uranium speciation was determined using KPA, XPS, XANES, and EXAFS analysis.

Chemical Characterization of FRC Groundwater FW-024

Sample	pH	Al	Ca	Mg	U
		---------- ppm ----------			
FRC data	3.7	611	1260	201	52
“as received”	3.4	471	897	155	43.8
pH adjusted	6.5	9.9	834	148	<0.5

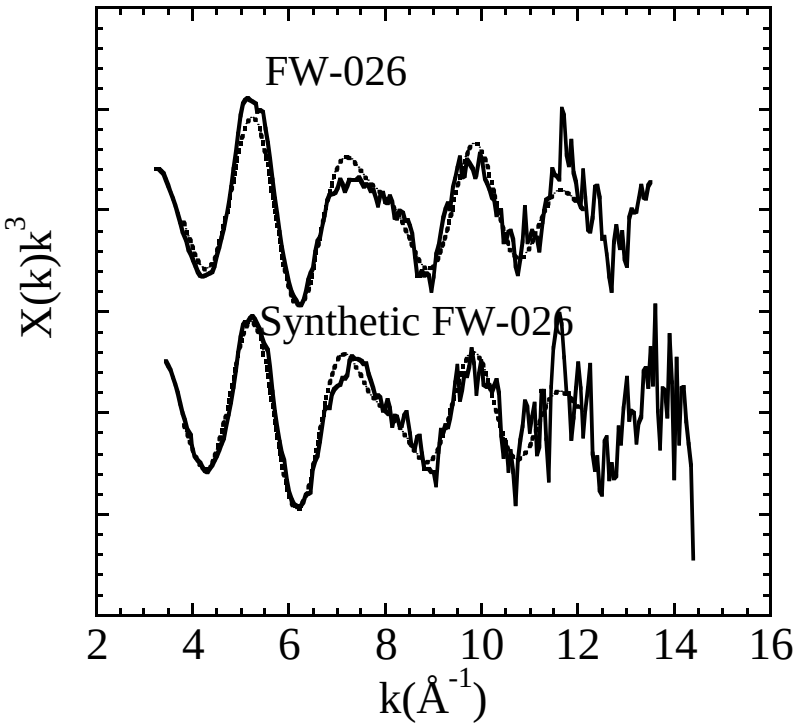
The pH of the groundwater was acidic and major metals were Al, Ca, Mg, and U. Adjustment of the groundwater to pH 6.5 resulted in precipitation of Al and U. Ca and Mg were only slightly affected.

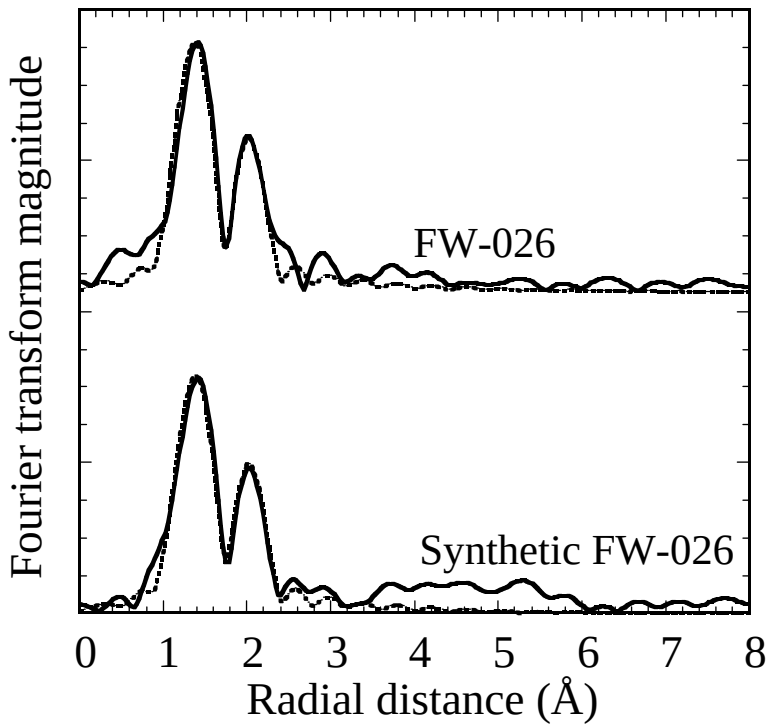
Potentiometric Titration of FRC Groundwater



Potentiometric titration of FW-024 groundwater shows presence of a buffer region between pH 4 and 5 due to formation of Al-hydroxide species. A whitish precipitate is formed at pH 6.5.

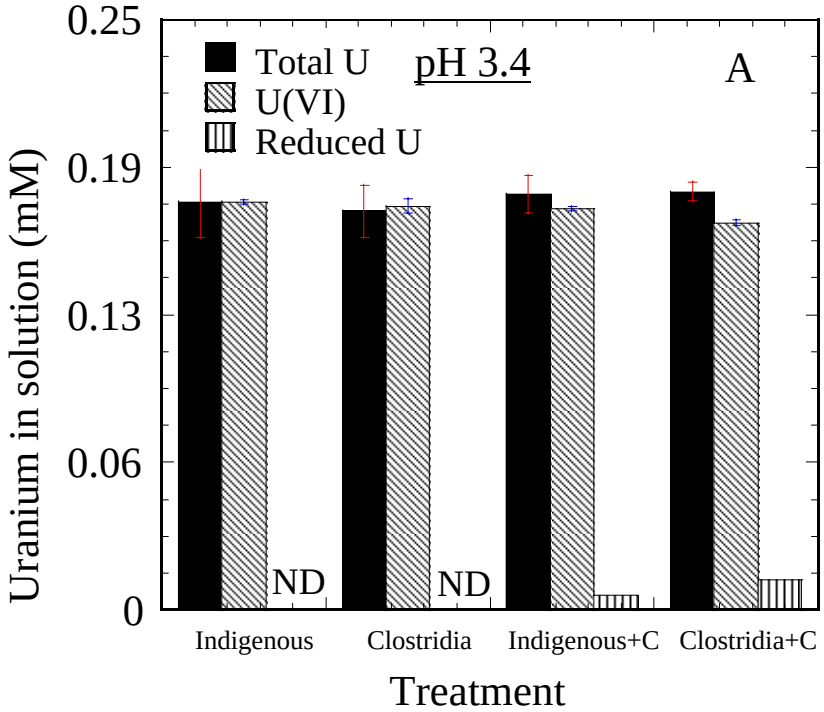
EXAFS Analysis of pH-Adjusted Groundwater

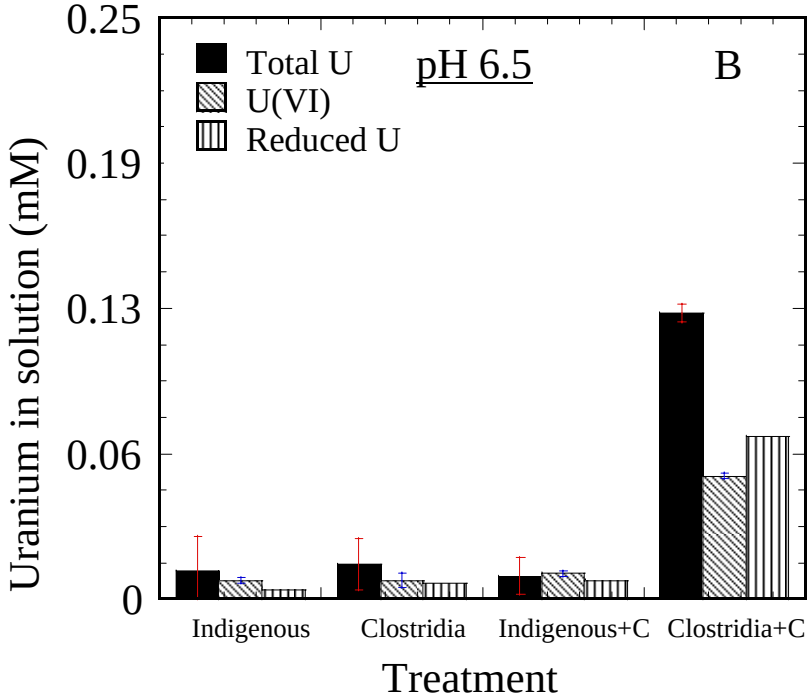




Comparison of pH-adjusted groundwater with synthetically prepared Al-U coprecipitate shows uranyl ion present as hydrated form and not associated as an inner-sphere complex with Al.

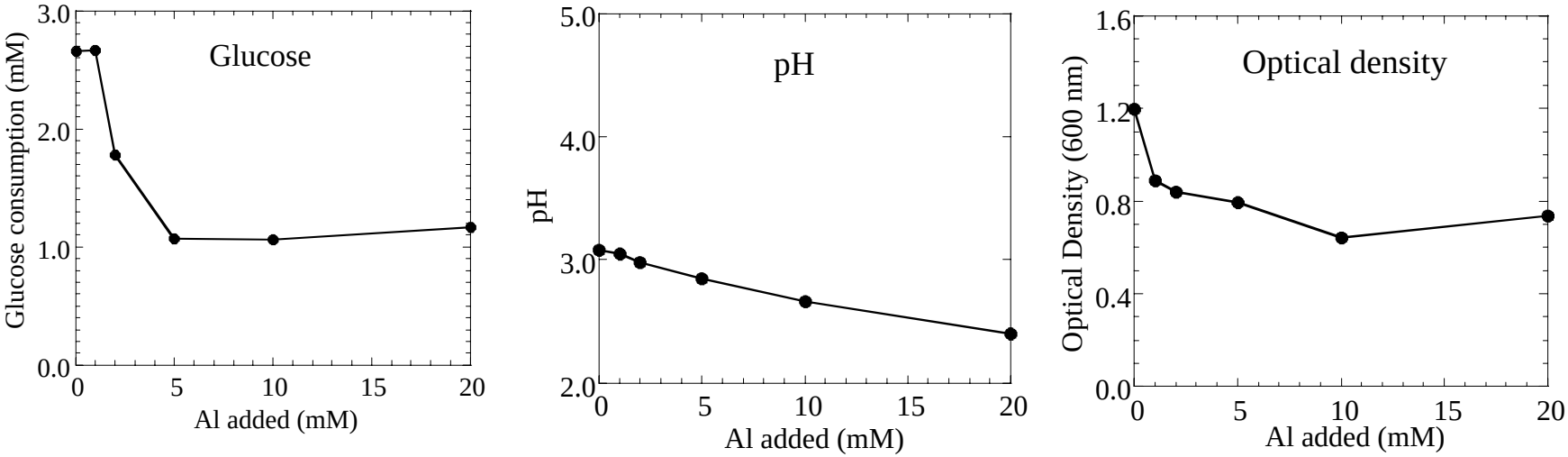
Effect of Indigenous Bacterial Growth on Uranium Reduction





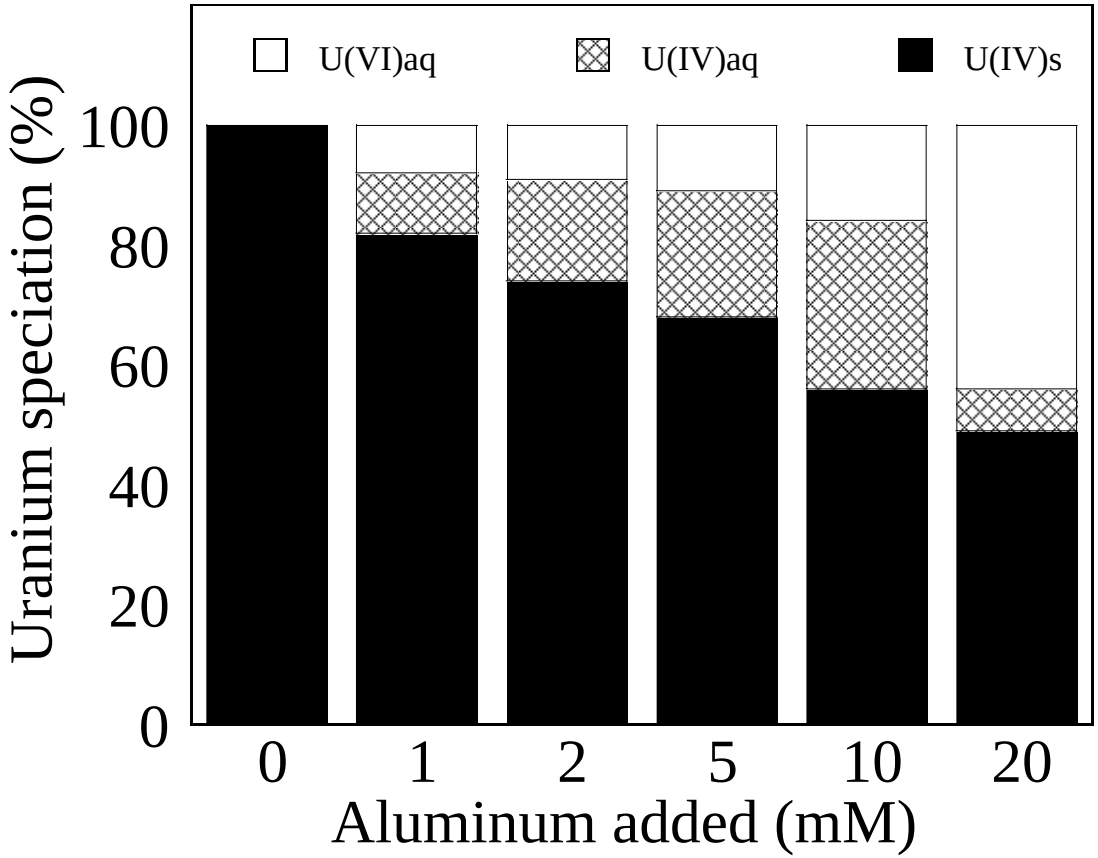
Uranium reduction at pH 3.5 was detected only in the presence of carbon source and indigenous or *Clostridium* sp. However, in the pH adjusted samples the added *Clostridium* sp. and glucose reduced 62% of the uranium to U(IV).

Effect of Aluminum Addition on Growth of Clostridium sp.



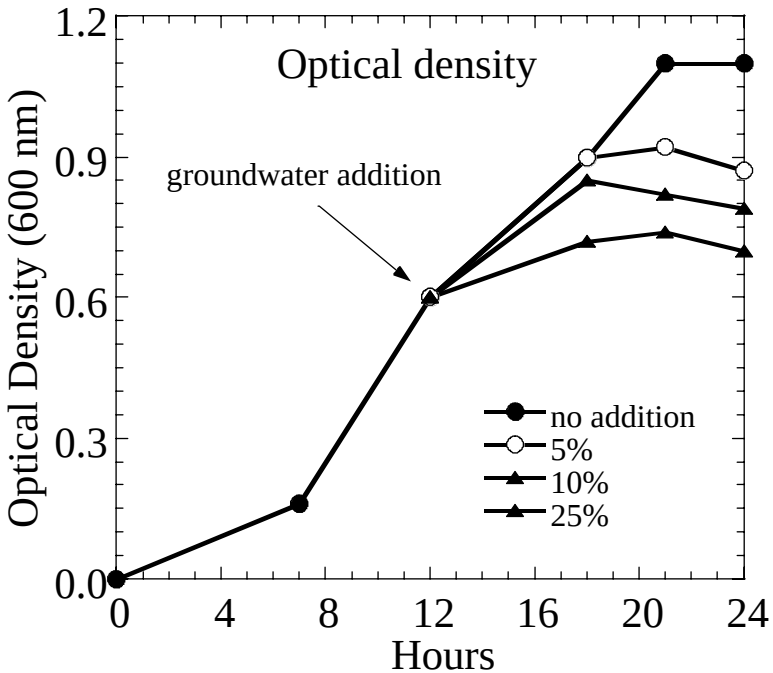
Addition of 1 to 20 mM aluminum to 12-hour old culture of *Clostridium* sp. inhibited growth of bacteria. The inhibition was most notable at >2 mM Al addition. This may be due to toxicity or low pH of the medium.

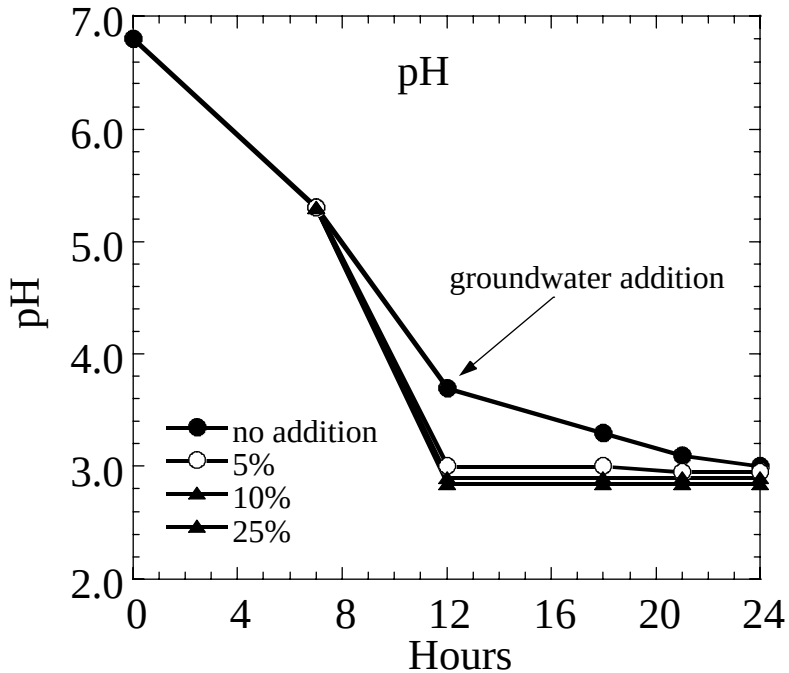
Effect of Aluminum Addition on Uranium Reduction by *Clostridium* sp.



Even though bacterial growth was inhibited, bacterial cells were able to reduce U(VI) to U(IV). The partitioning of uranium into the solid phase with increased Al concentration may be due adsorption to Al hydroxide colloids.

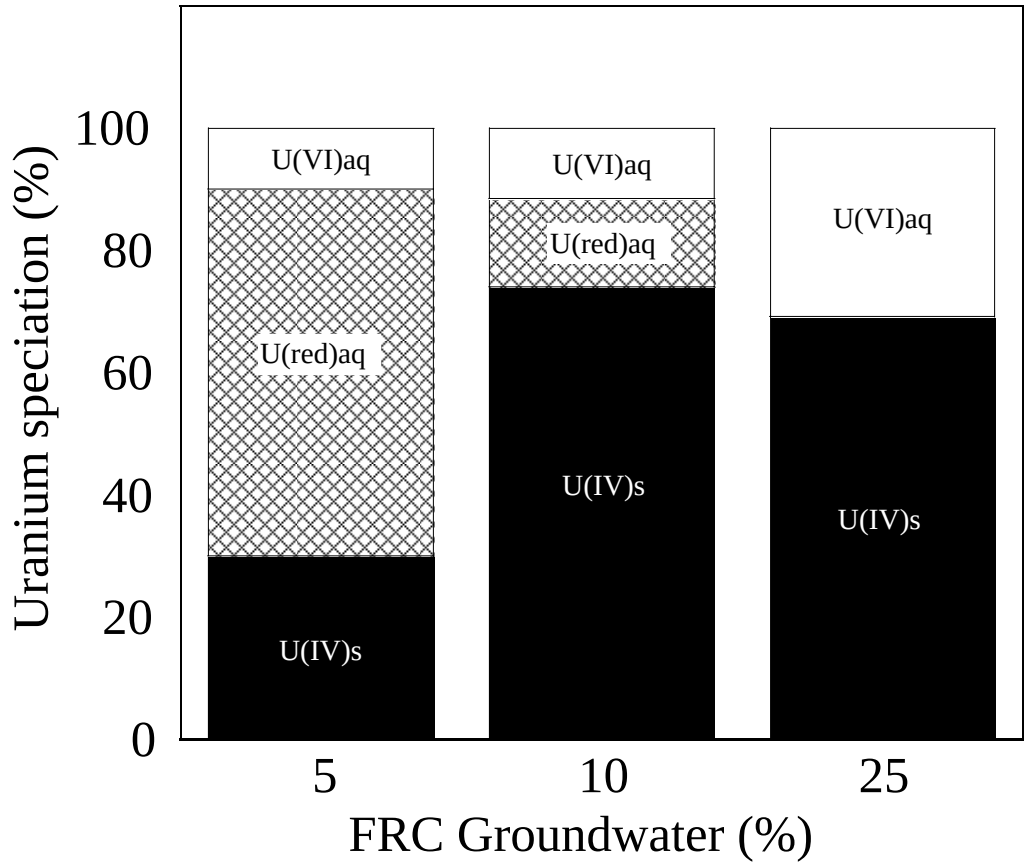
Effect of Addition of FRC Groundwater on Growth of *Clostridium* sp.





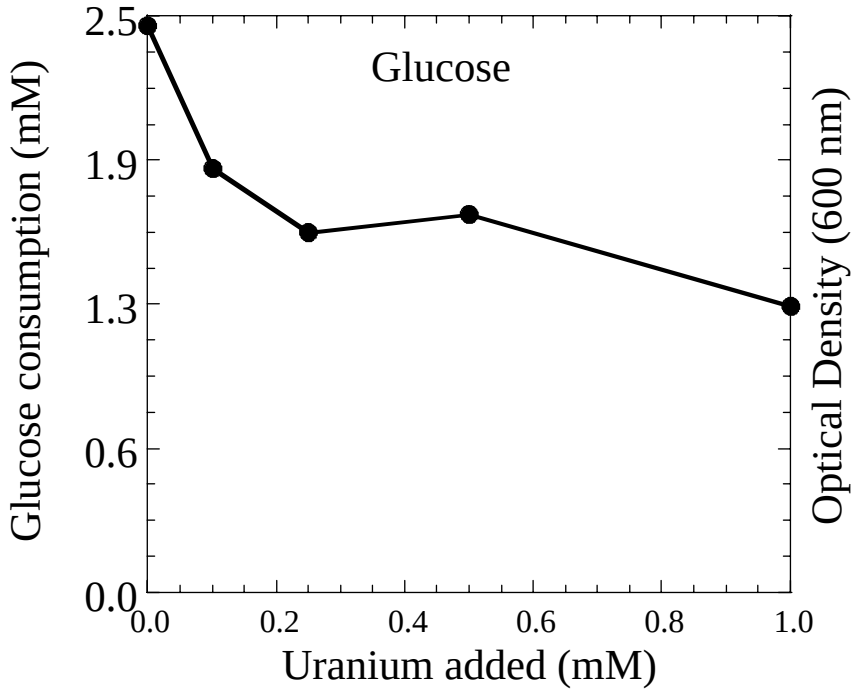
Addition of varying amount of FRC groundwater to 12 hour old culture of *Clostridium* sp. affected the growth of the bacteria as indicated by a decrease in optical density and pH with increasing groundwater addition.

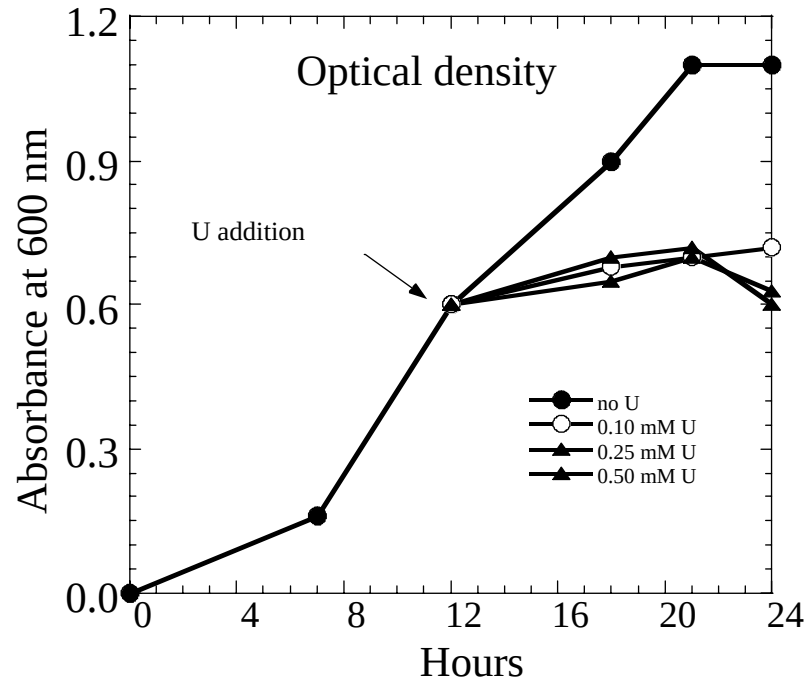
Speciation of Uranium Following Addition of FRC Groundwater to Clostridium sp.



A significant amount of uranium reduction to U(IV) was observed in up to 25% FRC groundwater.

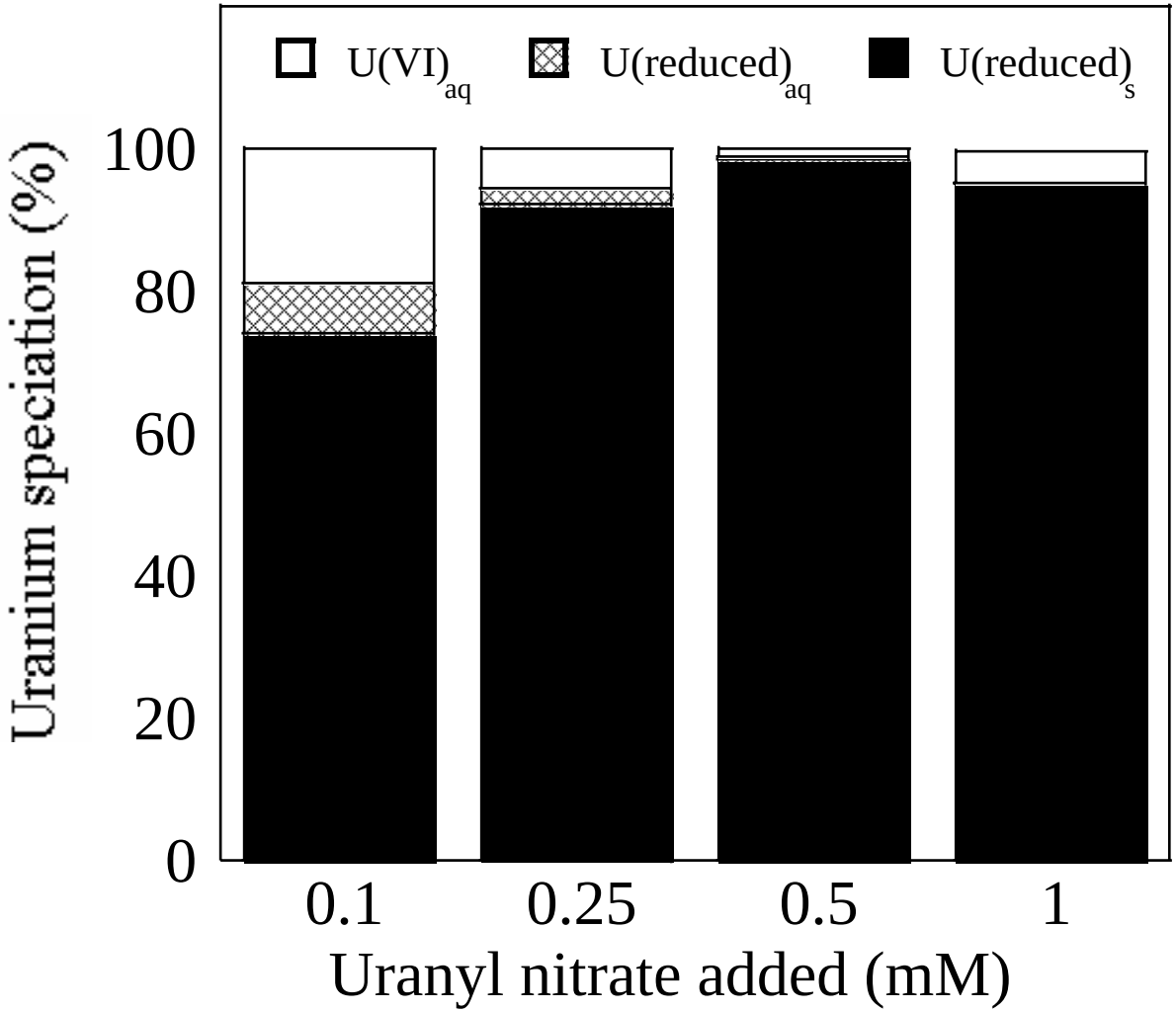
Effect of Addition of Uranyl Nitrate on Growth of Clostridium sp.





Addition of uranyl nitrate to 12 hour old culture of *Clostridium* sp. affected the growth as shown by decrease in optical density and glucose consumption.

Effect of Uranyl Nitrate on Uranium Reduction by *Clostridium* sp.



Although bacterial growth was inhibited, uranium reduction to U(IV) from U(VI)-nitrate occurred in the presence of up to 1.0 mM uranyl nitrate. The reduced uranium was present predominantly in the solid phase.

Summary

Pure culture studies:

- *Clostridium* sp. and *Clostridium sphenoides* reduced U(VI) to U(IV), Fe(III) to Fe(II), Tc(VII) to Tc(IV), and Pu(IV) to Pu(III).

- Uranium was reduced only in the presence of actively growing bacterial cells indicating a direct mechanism is involved.

FRC:

- In groundwater experiments slight reduction of U (3%) was observed at pH 3.4. At higher pH indigenous bacteria plus glucose reduced 6% of the U, while addition of *Clostridium* sp. plus glucose resulted in 62% uranium reduction.

- The primary mechanism of uranium reduction by *Clostridium* sp. in FRC groundwater appears to be the result of direct enzymatic activity.

Acknowledgements

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