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CITRATE BIODEGRADATION

A. J. Francis, C. J. Dodge, S. Chatterjee, and M. F. Landrey

Mid-year Status Report

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**BROOKHAVEN NATIONAL LABORATORY
UPTON, LONG ISLAND, NEW YORK 11973**

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**Citrate Biodegradation
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A. J. Francis, C. J. Dodge, S. Chatterjee, and M. F. Landry

**Department of Applied Science
Brookhaven National Laboratory
Upton, NY 11973**

Submitted to

M. Malone

**Program Manager, Uranium Soils ID
Office of Technology Development
Office of Environmental Management
US Department of Energy
Washington, DC**

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INTRODUCTION

The Uranium Soils Integrated Demonstration (USID) Program was established to demonstrate advanced technologies for the remediation of uranium contaminated soils. This program, managed by the Fernald Environmental Restoration Management Corporation (FERMCO), focuses on the development and demonstration of new soil remediation processes which are faster, safer, and more economical for use at the Fernald site and throughout the DOE complex for cleanup of similar contaminants [1].

Large-scale methods previously devised to deal with the problem of contaminated soils use corrosive reagents such as hot sulfuric or hydrochloric acids, and oxidizing agents such as sodium hypochlorite, to extract the metals. These methods not only cause irreparable damage to the soil, but also generate secondary waste streams which create further hazardous waste disposal problems. We, at Brookhaven National Laboratory (BNL) have developed a process which uses citric acid to treat contaminated soils and wastes with the subsequent recovery of toxic metals and uranium [2].

Citric acid, a naturally occurring organic chelating agent, forms multidentate stable complexes with the transition metals and actinides, and has been effectively used to extract uranium and other metals such as Al, Ba, Be, Cd, Co, Cr, Mn, Ni, Sr, Th, Zn, and Zr from solid wastes via the formation of soluble metal citrate complexes [3]. Further studies have also shown that several of the metal citrate complexes, such as Fe-, Zn-, and Ni-citrate, are readily biodegraded by microorganisms [4,5]. During this process, the metals form a precipitate or become associated with the biomass and are recovered at the end of the biodegradation [4,6]. Although uranyl citrate is recalcitrant to biodegradation, upon exposure to visible light it undergoes photochemical degradation resulting in the formation of an insoluble, stable polymeric form of uranium [7].

The BNL Citric Acid Process has also been effectively used to remove and separate toxic metals and radionuclides from sludge at the Y-12 Plant, Oak Ridge, TN, and toxic metals, including lead, from incinerator bottom ash [2,3,8].

Overall Objective

The overall objective of this study is to demonstrate the feasibility of the BNL Citric Acid Process to decontaminate uranium contaminated soils. The process is designed to recover uranium and the other metals separately, and in concentrated form for recycle and/or disposal.

Specific Objective

The specific objective of this study is to test the applicability of the BNL patented Citric Acid Process to treat effluents containing uranium and toxic metals generated from citric acid extractions of Fernald soils. This work is coordinated by Fernald Environmental Restoration Management Corporation (FERMCO) and Oak Ridge National Laboratory (ORNL) in an effort to clean up uranium contaminated soils at DOE sites.

In this process, the citric acid extract is first subjected to biological treatment to remove the excess citric acid, iron, and other metals. This is followed by photodegradation to remove uranium as insoluble uranium trioxide.

MATERIALS AND METHODS

Chemicals

All chemicals used in this study were of analytical grade or better and were purchased from Aldrich Chemical Company, Milwaukee, WI; Sigma Chemical Company, St. Louis, MO; or J. T. Baker, Phillipsburg, NJ. Standard solutions used for ICP-MS analysis were obtained from SPEX Industries, Inc., Edison, NJ.

Preparation of Citric Acid Extract

The extract was prepared by M. Timpson and C. W. Francis, ORNL, from contaminated Storage Pad Soils, Fernald, Ohio. Typically, 400 g of air dried soil was mixed overnight with 4 liters of 0.3 M citric acid using a magnetic stirrer. The solid impurities were removed by flocculation and filtration through a coarse filter paper [9]. The initial

uranium content of the soil was estimated to be 449 ppm and about 99% of uranium was extracted by the citric acid method [1]. Approximately 4 liters of extract were obtained in two separate plastic bottles. Upon receipt of the extract, it was stored at 4°C in the dark.

Characterization of Extract

The extract was characterized prior to experimentation. The analyses included determination of pH, citric acid, and metal content. The first bottle was analyzed when received (2/8/94), the second bottle a month later (3/5/94), and the remaining extracts from the two different bottles were combined and re-analyzed (5/3/94).

A white precipitate was formed during storage of the extract. The precipitate was separated from the liquid by filtration, air dried, and analyzed for citric acid and metals.

Citric Acid Analysis

Citric acid was analyzed using high pressure liquid chromatography (HPLC) (Shimadzu LC-10AS) equipped with a UV-vis detector (SPD-10A) set at 210 nm. The mobile phase was 0.003 N H₂SO₄ at a flow rate of 0.6 ml/min. For both standard and samples, 50 µl was injected onto a 300 mm x 7.8 mm Aminex HPX-87H column, (Bio-Rad, Hercules, CA). Identification of citric acid was made by comparison of peak retention time with that of a standard compound. Citric acid concentration was calculated from the sample peak area by comparison with a standard curve. The analytical precision (± 1 S.D.) of replicate injections was 5%.

Metal Analysis

Metals were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS). Liquid samples were diluted with 1% Ultrex HNO₃ (J. T. Baker, Philipsburg, NJ), and solid samples were subjected to acid digestion before analysis. A portion of the white precipitate obtained from the ORNL extract (0.5 g) was dissolved in 5 ml of concentrated Ultrex HNO₃, diluted to 100 ml with deionized water, and then analyzed. Indium was added as an

internal standard, and the concentration for each metal was calculated based on a standard curve.

Biodegradation of Extract

Effect of extract concentration on biodegradation. A *Pseudomonas fluorescens* biovar II (ATCC #55241), capable of degrading citric acid as sole carbon source, was grown in a modified Simmon's citrate medium containing (g/l): citric acid, 2.0; MgSO₄, 0.2; NH₄Cl, 1.0; K₂HPO₄, 1.0; and NaCl, 5.0. The pH of the medium was adjusted to 6.0 with NaOH. The bacterium was cultured at 26° ± 1°C in the dark on a rotary shaker. To determine the optimum extract concentration for the citric acid degradation, three concentrations of the citric acid extract were prepared and subjected to an aerobic biodegradation. The treatment consisted of i) undiluted extract; ii) extract diluted two times; and iii) extract diluted four times.

The citric acid extract was filtered using a 0.45 µm sterile disposable filtration unit (Corning, Corning, NY). The pH of the extract was adjusted to 6.0 with NaOH pellets. Duplicate samples for each concentration of the extract were amended with nutrients consisting of 0.1% (w/v) NH₄Cl, K₂HPO₄, and KH₂PO₄. Two hundred milliliters of the sample was then placed in a 500-ml flask in duplicate and inoculated with 7 ml of an 18-h old culture of *P. fluorescens*. The flasks were fitted with cotton plugs and incubated on a rotary shaker at 24 ± 1°C in the dark. Uninoculated control samples in duplicate, were also included.

All sample preparation was performed under low light to minimize possible photodecomposition of the metal-citrate complexes. During incubation, 5 ml aliquots were periodically taken and filtered through a 0.22 µm Millex filter (Millipore, Bedford, MA). The filtered samples were analyzed for pH, citric acid, uranium and metals as described above.

Effect of nutrient addition on biodegradation. An experiment was also conducted to compare the effect of nutrient addition (N and P) on the biodegradation of citric acid. This experiment was performed with undiluted extract and followed the same procedure as described above, except a 0.5% nutrient addition was used instead of 0.1%.

Recovery of Metals

At the end of the biodegradation experiment, the samples were centrifuged (6000xg, 25 min) to separate the biomass from solution. The supernatant was used for the photodegradation experiment and the solid phase (biomass) was dried at 70°C overnight, weighed, digested with Ultrex HNO₃, diluted to 100 ml, and analyzed for metals.

Photodegradation of Extract

The supernatant after biodegradation of the fourfold diluted extract was subjected to photodegradation. In order to avoid microbial activity during photodegradation, the supernatant was filtered through a 0.45 µm sterile filter to remove any bacteria remaining after centrifugation.

The photodegradation experiments were carried out at two different pHs. The supernates were combined and then divided into two equal volumes. One was adjusted to pH 3.5 and the other to pH 6.0. One hundred milliliters of the sample were transferred to 250 ml flasks. The flasks were fitted with cotton plugs and exposed to seven 60 watt, high output fluorescent growth lights at a distance of 20 cm in an incubator. The light intensity during photodegradation was 0.27 mEinstein m⁻² sec⁻¹. All photochemistry reactions were set up in triplicate and a control experiment was run simultaneously in the dark. Periodically, 5 ml aliquots were withdrawn from each flask and the pH was measured. These samples were filtered through 0.22 µm Millipore filters and analyzed for citric acid and uranium concentration. At the end of the experiment, the samples were centrifuged at 9000xg to recover the precipitate.

Table 1. Characterization of Fernald Storage Pad Soil Extract

	(2/8/94)	Analysis Date (3/5/94)	(5/3/94)
pH	3.2	3.1	3.0
Citric acid (M)	0.35	0.28	0.22
<i>Metals (mg/l)</i>			
Aluminum (Al)	90	102	101
Barium (Ba)	3.8	4.0	3.3
Calcium (Ca)	3910	1260	98
Cobalt (Co)	NA	NA	0.4
Chromium (Cr)	0.15	0.15	0.10
Copper (Cu)	0.9	0.9	0.7
Iron (Fe)	63	64	98
Lead (Pb)	0.04	0.03	0.02
Magnesium (Mg)	872	1120	830
Manganese (Mn)	44	45	37
Nickel (Ni)	1.2	1.5	1.3
Strontium (Sr)	4.1	3.7	2.9
Thorium (Th)	NA	NA	0.1
Titanium (Ti)	8.4	6.0	5.5
Uranium (U)	42	38	31
Vanadium (V)	NA	NA	0.2
Zinc (Zn)	0.75	1.5	1.0

NA = not analyzed

Mass Balance for Metals

A mass balance was done to determine the efficiency of metal removed during biodegradation and photodegradation. The balance was based on the initial metal concentration of the extract and the final metal concentration in the liquid and solid phases.

Table 2. Characterization of Precipitate in Fernald Storage Pad Soil Extract

<i>Major Components (% dry weight)</i>	
Calcium (Ca)	16.1
Citric acid	76.8
<i>Metals (µg/g dry weight)</i>	
Aluminum (Al)	354
Barium (Ba)	35
Chromium (Cr)	<5
Copper (Cu)	<5
Iron (Fe)	412
Lead (Pb)	19
Magnesium (Mg)	2230
Manganese (Mn)	148
Nickel (Ni)	6
Strontium (Sr)	184
Titanium (Ti)	639
Uranium (U)	147
Zinc (Zn)	20

RESULTS AND DISCUSSION

Characterization of Extract and Precipitate

In Table 1, the composition of the Fernald Storage Pad Soil Citric Acid Extract stored at 4°C is presented as a function of time. Based on the extraction information provided by ORNL, the uranium concentration of the citric acid extract is estimated to be about 40mg/l, which is in good agreement with the measured values. There is significant reduction in concentration of citric acid (37%) and Ca (97%) over a period of three months. The uranium concentrations in the time period decreased from 42 to 31 mg/l.

The chemical characteristics of the precipitate recovered from ~ 4 L of citric acid extract is given in Table 2. The approximate weight of the precipitate was 110 g. The bulk of the precipitate was composed of Ca and citric acid. The weight ratio of citric acid to calcium was 4.8 indicating the presence of 1:1 mole ratio of the Ca citrate salt. The

presence of large amounts of Ca in the extract results in formation of a supersaturated solution of calcium citrate. Over a period of several months a calcium citrate salt is formed which slowly precipitates from solution. Our experience with this process has been that extracts which contain significant amounts of calcium should be treated soon after extraction. This will prevent formation of a precipitate since Ca will remain in solution in ionic form after biodegradation. The precipitate also contained small amounts of the following metals: Al, Ba, Fe, Pb, Mg, Mn, Sr, Ti and U.

Biodegradation

Figure 1 shows the citric acid degradation at various dilutions. The initial citric acid concentration in the 4 times diluted sample was 75 mM, and was degraded completely in 7 days at a rate of 0.45 mM/h, whereas only 64% and 35% of the citric acid was degraded in 10 and 12 days for the 2 times and the undiluted samples, respectively. The degradation

Figure 1. Effect of Initial Citric Acid Concentration on the Biodegradation of Extract.

Figure 2. Change in pH During the Biodegradation of soil Extract.

Figure 3. Degradation of Citric Acid and Removal of Uranium During Biodegradation of 4 Times Diluted Soil Extract.

rate for the 2 times dilution was 0.38 mM/h while the undiluted sample started at 1 mM/h, but remained relatively constant after 4 days at 0.03 mM/h. Since the 4 times dilution gave complete citric acid degradation, this dilution was used in subsequent experiments. The metals present in the citric acid extract did not appear to inhibit bacterial growth as shown by the degradation of citric acid.

During biodegradation, the pH increased steadily from the initial value of 6.3. The increase was greater for the 4 and 2 times diluted samples than for the undiluted. After 24 hours, the pH of the 4 and 2 times diluted sample was 9.2 and 9.0, respectively, while the pH of the undiluted sample increased to 8.9 after 10 days (Figure 2).

Figure 3 shows the degradation of citric acid and the concentration of uranium in 4 times diluted citric acid soil extract. Citric acid was completely degraded in 92 hours at a rate of 100 ppm/hr while the uranium decreased from 8.0 to 6.1 ppm after 138 hours.

The metal removal during the biodegradation for the 4 times diluted samples is presented in Figure 4. The concentration of metal in the extract generally decreased within the first few days of incubation.

Figure 4. Changes in Concentrations of Metals During Biodegradation of Extract.

Table 3. Effect of Biodegradation in the Treatment of Citric Acid Extract

Metal	Before Biodegradation (mg/l)	After Biodegradation (mg/l)	Removal Efficiency (%)
Al	21.5 ± 0.5*	0.307 ± 0.019	99
Ba	0.784 ± 0.017	0.269 ± 0.192	66
Ca	23.3 ± 0.2	1.19 ± 0.13	95
Co	0.095 ± 0.010	0.044 ± 0.003	54
Cr	0.055 ± 0.023	0.016 ± 0.005	71
Cu	0.221 ± 0.029	0.067 ± 0.011	70
Fe	24.1 ± 0.5	< 0.001	> 99
Mg	178 ± 5	58.9 ± 2.4	67
Mn	9.33 ± 0.17	0.055 ± 0.003	99
Ni	0.483 ± 0.109	0.250 ± 0.044	48
Pb	0.022 ± 0.004	0.001 ± 0.000	95
Pt	0.193 ± 0.137	0.003 ± 0.001	99
Sc	0.034 ± 0.000	< 0.001	99
Sr	0.696 ± 0.026	0.004 ± 0.002	99
Th	0.034 ± 0.003	0.004 ± 0.000	88
Ti	1.36 ± 0.05	0.074 ± 0.006	95
U	8.06 ± 0.13	6.07 ± 0.21	25
V	0.073 ± 0.007	0.080 ± 0.004	--
Zn	1.40 ± 0.79	0.740 ± 0.517	47

* ± 1 SEM.

Metal removal during a typical biodegradation experiment is summarized in Table 3. The removal efficiency for Al, Ca, Fe, Mn, Pb, Pt, Sc, Sr, and Ti was ≥ 95%; Cu (70%); Mg (67%); Ni (48%); and Th (88%). A small amount of U (25%, 1.9 ppm) was removed from solution during biodegradation.

The concentration of metals associated with the biomass after biodegradation is given in Table 4. The biomass is enriched in Al, Ca, Fe, Mg, Mn, Ti, and U at the end of biodegradation.

Table 4. Metals Associated with Biomass after Biodegradation of Citric Acid Extract

Metal	$\mu\text{g/gdw}$
Al	8674
Ba	288
Bi	0.60
Ca	7651
Co	9.40
Cu	26.3
Fe	9581
Mg	32093
Mn	3558
Ni	55.3
Pb	7.21
Sc	15.6
Sr	263
Th	10
Ti	519
U	563
V	10.6
Zn	223
Zr	13.4

The dried precipitate comprising both metals and microbial cells weighing 0.43 g was recovered from 200 ml of 4 times diluted extract. Typically, 0.2 gdw of solids produced per gram of citric acid degraded.

Photodegradation

The photodegradation of citric acid extract after biodegradation at pH 3.5 is shown in Figure 5. The photodegradation at pH 6.0 did not result in significant uranium precipitation. Table 5 gives the metal contents of the precipitate formed during photodegradation. The amount of precipitate recovered was 0.02 gdw per 150 ml of the extract. The removal efficiency of uranium by photodegradation in extract which has been previously subjected to biodegradation is shown in Table 6. The concentrations of other

Table 5. Analysis of Precipitate after Photodegradation of Citric Acid Extract

Metal	$\mu\text{g/gdw}$
Al	27
Ba	65
Ca	1570
Co	1.5
Cu	835
Fe	258
Mg	7150
Mn	18.5
Ni	21
U	8600
Zn	120

Figure 5. Removal of Uranium During Photodegradation of Extract.

Table 6. Effect of Photodegradation in the Treatment of Extract

Metal	Before photodegradation (mg/l)	After Photodegradation (mg/l)	Removal Efficiency (%)
Al	0.405 $\pm$ 0.026*	0.408 $\pm$ 0.000	-
Ca	3.68 $\pm$ 0.46	4.84 $\pm$ 0.12	-
Co	0.062 $\pm$ 0.001	0.056 $\pm$ 0.005	-
Cu	0.130 $\pm$ 0.008	0.159 $\pm$ 0.026	-
Fe	< 0.001	0.042 $\pm$ 0.030	-
Mg	85.6 $\pm$ 1.1	83.1 $\pm$ 0.3	-
Mn	0.095 $\pm$ 0.003	0.099 $\pm$ 0.000	-
Ni	0.213 $\pm$ 0.001	0.193 $\pm$ 0.020	-
U	5.93 $\pm$ 0.01	1.63 $\pm$ 0.01	72.5
V	0.079 $\pm$ 0.005	0.071 $\pm$ 0.000	-
Zn	0.040 $\pm$ 0.015	0.019 $\pm$ 0.007	-

* $\pm$ 1 SEM.

metals detected in solution were very low. The remainder of the precipitate most probably consisted of organic matter from the biodegradation step which was removed during digestion of the precipitate.

The overall removal efficiency after bio- and photo-degradation of the Fernald Storage Pad Soil citric acid extract is given in Table 7. Ba, Bi, Fe, Ir, Mn, Mo, Pt, Sr, Th, Ti, and Zn were removed $\geq 99\%$, and Al, Pb, Sc, and Zr $> 90\%$. The removal efficiency of uranium was 80%.

Uranium Mass Balance

Mass balance calculations show that 25% of the uranium was recovered in the biomass after biodegradation, and 72.5% of that remaining after biodegradation was recovered by photodegradation. The uranium content was reduced from an initial 8.06 ppm to 1.63 ppm with a total removal efficiency of 80%.

**Table 7. Effect of Biodegradation Followed by Photodegradation
in the Treatment of Citric Acid Extract.**

Metal	Extract (mg/l)	After Biodegradation (mg/l)	After Photodegradation (mg/l)	Overall Removal Efficiency (%)
Al	21.5 ± 0.5*	0.307 ± 0.019	0.408 ± 0.000	98
Ba	0.784 ± 0.017	0.269 ± 0.192	0.004 ± 0.003	> 99
Bi	0.005 ± 0.005	< 0.001	< 0.001	> 99
Ca	23.3 ± 0.2	1.19 ± 0.19	4.84 ± 0.12	77
Co	0.095 ± 0.010	0.044 ± 0.003	0.056 ± 0.005	36
Cr	0.055 ± 0.023	0.016 ± 0.005	0.019 ± 0.002	75
Cu	0.221 ± 0.029	0.067 ± 0.011	0.159 ± 0.026	47
Fe	24.1 ± 0.5	< 0.001	0.042 ± 0.030	> 99
Ir	0.014 ± 0.005	0.001 ± 0.001	< 0.001	> 99
Mg	178 ± 5	58.9 ± 2.4	83.1 ± 0.3	52
Mn	9.33 ± 0.17	0.055 ± 0.003	0.099 ± 0.000	99
Mo	0.014 ± 0.005	0.016 ± 0.003	< 0.001	> 99
Ni	0.483 ± 0.109	0.250 ± 0.044	0.193 ± 0.020	65
Pb	0.022 ± 0.004	0.001 ± 0.000	0.093 ± 0.148	95
Pt	0.193 ± 0.137	0.003 ± 0.001	< 0.001	> 99
Sc	0.034 ± 0.000	< 0.001	0.003 ± 0.001	94
Sr	0.696 ± 0.026	0.004 ± 0.002	0.003 ± 0.003	> 99
Th	0.034 ± 0.003	0.004 ± 0.000	< 0.001	> 99
Ti	1.36 ± 0.05	0.074 ± 0.006	< 0.001	> 99
U	8.06 ± 0.13	6.07 ± 0.21	1.63 ± 0.01	80
V	0.073 ± 0.007	0.080 ± 0.004	0.071 ± 0.000	--
Zn	1.40 ± 0.79	0.740 ± 0.517	0.019 ± 0.007	> 99
Zr	0.078 ± 0.000	0.004 ± 0.001	0.006 ± 0.001	94

* ± 1 SEM.

CONCLUSIONS

1. Analysis of biodegradation and photodegradation experiments initially conducted on the Fernald Storage Pad Soil Extract indicate that uranium and other metals, including Al, Ba, Bi, Ca, Cr, Fe, Ir, Mg, Mn, Mo, Ni, Pb, Pt, Sc, Sr, Th, Ti, Zn, and Sr can be effectively removed using the BNL Citric Acid Process.
2. The selective removal of uranium from the toxic metals in the extract is accomplished by photodegradation.

RECOMMENDATIONS/FUTURE WORK

Currently, the biodegradation and photodegradation studies are being repeated with the ORNL Fernald Storage Pad Soil citric acid extract using the BNL Citric Acid Process to confirm the applicability of the treatment process.

I. Enzymatic Degradation of Citric Acid Extract

Degradation of citric acid and metal citrate complexes by enzymatic action including immobilized enzymes will be tested. This process should eliminate the biomass buildup and photodegradation step, and precipitation of metals, and U in a single step.

II. Recovery/Recycle of Citric Acid

Development of a process for recovery of free citric acid from the extract prior to biodegradation. Some of the strategies investigated will include: solvent extraction, precipitation, and chromatographic separation.

Successful design of a recycling scheme will decrease the costs of raw materials and hence make the process more economically viable. The schematic representation of the proposed integrated remediation process is presented in Figure 6.

Figure 6. Schematic Representation of Integrated Citric Acid Remediation Process.

III. Optimization of Process Parameters

Biodegradation

1. At present, all biodegradation experiments are conducted in 500 ml batch reactors with a 200 ml working volume. The processing volume will be scaled up to 1 liter and all biodegradations will be carried out in 2 liter continuous stirred tank reactors (CSTRs).
2. Optimization of process parameters such as pH, temperature, mixing speed, and oxygen mass transfer rate will be carried out to maximize both reaction rates and overall conversions.
3. The CSTRs will also be run in a continuous mode to investigate the possibility of continuous operation.

Photodegradation

1. Optimization of process parameters such as temperature, light intensity, and wavelength (e.g. UV) will be carried out to maximize both reaction rates and overall conversions.
2. The possibility of increasing the rates and conversion of uranium precipitation via the addition of nucleation agents as well as addition of catalysts will be investigated.

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