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**Radiotoxicity of Neptunium(V) and Neptunium(V) - Nitrilotriacetic (NTA) Acid Complexes
Towards *Chelatobacter heintzii***

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Abstract

1 The objective of this work was to investigate the toxicity mechanisms of neptunium and the
2 neptunium-NTA complex towards *Chelatobacter heintzii*. Our results show that metal
3 toxicity of aquo NpO_2^+ may significantly limit growth of *C. heintzii* at free metal ion
4 concentrations greater than $\approx 10^{-5}$ M. However, neptunium concentrations $\geq 10^{-4}$ M do not
5 cause measurable radiotoxicity effects in *C. heintzii* when present in the form of a
6 neptunium-NTA complex or colloidal/precipitated neptunium-phosphate. The neptunium-
7 NTA complex, which is stable under aerobic conditions, is destabilized by microbial
8 degradation of NTA. When phosphate was present, degradation of NTA led to the
9 precipitation of an neptunium-phosphate phase.

Introduction

Past practices at U.S. Department of Energy (DOE) facilities have resulted in the contamination of subsurface aquifers with mixtures of organic compounds and radionuclides. An important class of mixed contaminants includes co-disposed actinides and organic chelating agents, such as nitrilotriacetic acid (NTA) (27). The presence of strong complexing agents in subsurface environments can increase dissolved actinide concentrations, leading to enhanced radionuclide migration in groundwater (20).

The extent of NTA-induced migration depends on the long-term stability of the actinide-chelate complex. It has recently become clear that microorganisms play an important role in subsurface environmental chemistry (4). Microbial activities affect the concentration of biodegradable compounds and redox conditions in subsurface aquifers. Furthermore, microbial degradation of primary substrates in many cases produces or consumes acid equivalents, lowering or raising system pH (28). Therefore, the chemical speciation of redox and acid/base sensitive compounds in groundwaters can be largely dependent on biological activities.

NTA is utilized by some bacteria, including *Chelatobacter heintzii*, as a sole source of carbon, nitrogen, and energy (8). Degradation of NTA by microorganisms decreases chelate availability, which modifies the speciation of metals and actinides in the system (3). The actinide species may form a complex with other ligands in the system; sorb onto oxides, organic matter surfaces, or bacteria cell membranes; or precipitate from solution (13, 29).

The speciation of actinides in the subsurface, subsequent to the microbiological degradation of the organic complexant, is defined by the inorganic (e.g., phosphate, carbonate, hydroxy) and organic (humic/fulvic acids) anions present, as well as pH. Of particular relevance to this paper, phosphates control metal solubility in some subsurface environments (30).

Morgenstern and Kim (22) determined formation constants for phosphate complexation with Np(V) and used chemical equilibrium calculations to predict that neptunium-phosphate complexation dominates carbonate complexation at phosphate concentrations $\geq 10^{-4}$ M. Bioprecipitation of uranium as a phosphate phase was linked to phosphatase-enzyme activity in a *Citrobacter* sp, suggesting that biologically mediated phosphate precipitation may be an effective method of removing actinides from contaminated waters (14-16).

Radioactive elements, such as actinides, are unique because they are potentially toxic to microorganisms via two distinct mechanisms. First, actinide species can be chemically toxic, similar to other metals. The uncomplexed metal ions are usually more toxic to microorganisms than complexed metal species (1, 6, 11, 17). Neptunium may inhibit growth in the same manner as other metals, for example by binding with proteins necessary for substrate transport or by substitution into cofactor metal ion sites (6). Second, microorganisms are susceptible to toxic effects caused by the ionizing radiation emitted during radioactive decay (9, 18, 34). Ionizing radiation induces toxicity by causing unrepairable cell damage that prevents cell reproduction or causes cell death via several potential pathways. Reed (26) found that radiotoxicity effects of plutonium were induced below the absorbed dose threshold due to intimate association of the plutonium with *C. heintzii*. Neptunium, primarily an alpha particle emitter (10), is potentially toxic to microorganisms if alpha particles emitted interact directly with the cell when the radionuclide is ingested by the organism during metabolic processes (34), is present in high enough concentrations to apply overall radiation doses exceeding the organism's radiation threshold (9), or is sorbed extracellularly (26).

As part of our continuing investigations of the interactions between actinide species and microorganisms, the objective of this work was to explore the potential toxicity mechanisms of

neptunium towards *C. heintzii* and establish the stability of the neptunium-NTA complex in the presence of aerobic microbiological activity. We found that only the free NpO_2^+ ion inhibited growth of *C. heintzii* and that the toxic effects were mitigated when neptunium was complexed with NTA or phosphate or was bound in a neptunium-phosphate colloid/precipitate.

METHODS AND MATERIALS

Aquo NpO_2^+ ion toxicity was determined by growing *C. heintzii* in a fixed-pH glucose media with various neptunium concentrations. The glucose minimal media was used to prevent significant complexation with neptunium. Growth in the glucose media was intentionally nutrient limited to measure potential short-term toxicity effects.

In order to determine the stability of the neptunium-NTA complex and the potential radiotoxicity of neptunium-NTA towards *C. heintzii*, variable-pH experiments were conducted in a defined mineral salts NTA growth medium and with varying neptunium concentrations. Growth- medium speciation during NTA degradation was computed using the dynamic chemical equilibrium computer code, CCBATCH (see Appendix A for a brief description of CCBATCH and the modeling methodology used for this work), developed at Northwestern University for the study of coupled chemical and biological processes (28). Chemical equilibrium calculations indicated that the initial dominant neptunium speciation in the medium was 63% $\text{NpO}_2\text{NTA}^{2-}$, 24% $\text{NpO}_2\text{HPO}_4^-$, 10% NpO_2^+ and 2% $\text{NpO}_2\text{PO}_4^{2-}$. Absorption spectroscopy verified that $\text{NpO}_2\text{NTA}^{2-}$ (990.2 nm) was the predominant neptunium species. $\text{NpO}_2\text{PO}_4^{2-}$ (988.8 nm) could not be initially identified spectroscopically due to peak convolution with $\text{NpO}_2\text{NTA}^{2-}$. Growth in the NTA experiments was limited only by the amount of NTA available as a primary substrate.

Initial cell inoculations and total neptunium concentrations for both sets of experiments are detailed in Table I.

Sample preparation. *Chelatobacter heintzii* (ATCC 29600), an obligately aerobic NTA degrader, was grown in a defined NTA culture media (31) consisting of the following mineral additions (in gm per L): K_2HPO_4 , 1.606; KH_2PO_4 , 0.403; NH_4NO_3 , 0.499; $MgSO_4 \cdot 7 H_2O$, 0.203; $CaCl_2 \cdot 2 H_2O$, 0.0200; $FeCl_3 \cdot 6H_2O$, 0.0027; NTA, 0.999; and NaOH to adjust was the pH to 7.0 ± 0.1 . Growing cells were harvested in the log phase of growth (600 nm Optical Density (OD_{600}) ≈ 0.1); "resting" cells were harvested in post-log phase ($OD_{600} > 0.1$). Cells were rinsed three times in $pH 6.2 \pm 0.1$ 0.01 M PIPES (Piperazine-N, N'-bis[2-ethanesulfonic acid]) solution, concentrated by centrifugation, and re-suspended in 0.01 M PIPES solution to prepare an inoculum with a final OD_{600} of 0.4-0.6.

Sterile neptunium-NTA sample vessels were prepared by adding incremental amounts of $\geq 95\%$ isotopic purity ^{237}Np stock solution, filtered through a 0.2- μm membrane filter, to defined mineral media in 30-ml serum bottles. Three control samples were prepared in a similar manner without addition of neptunium. All samples were inoculated with the culture described above.

Neptunium-glucose experiments were conducted in a minimal glucose growth medium to prevent neptunium-organic complexation. The medium was prepared by addition of 0.942 gm/l glucose (dextrose) to a 0.01M PIPES solution. The final pH of the solution was adjusted to 6.0 ± 0.1 before 0.2- μm membrane filter sterilization. Aquo $Np(V)$ samples were then prepared by adding incremental amounts of sterile neptunium stock. All growth experiments were performed in the dark at room temperature ($21 \pm 2^\circ C$) under aerobic conditions.

Sample analysis. 1-ml aliquots were periodically drawn from each vessel and analyzed for biomass production and neptunium-NTA complex stability by measuring the visible light-near infra red (VIS-NIR) absorption spectrum from 575 to 1250 nm using a Varian CARY 5

spectrophotometer. A 0.2-ml subsample was then drawn from each aliquot and added to 10 ml of Ultima Gold scintillation cocktail (Packard); another 0.1-ml subsample was added to 0.9 ml of sterile, deionized water. The remainder of the aliquot was filtered through a 0.2- μ m filter, and 0.2 ml of the filtered material was added to a second 10-ml scintillation cocktail. Total neptunium concentrations were determined by α -particle scintillation counting of the unfiltered preparation. The amount of bioassociated neptunium was calculated from the difference in α -particle scintillation counts between the unfiltered and filtered samples. All counting was done with a Packard model 2500 TR liquid scintillation analyzer.

The long term viability of *C. heintzii* in the presence of neptunium was monitored by Colony Forming Unit (CFU) plate counts. The ten-fold diluted subsamples described above were used to prepare serial dilutions up to 10^{-8} :1. Two 25 μ l splits from each dilution were spread onto 6% Agar-NTA growth media culture plates and observed for colony formation.

Extraction of particulate-associated neptunium was determined by two methods: via dilution by the addition of 0.1 ml of unfiltered sample to 0.9 ml deionized water, and via recomplexation by addition of 0.1 ml of unfiltered sample to 0.9 ml of 0.001 M NTA solution.

Colloidal/precipitate neptunium-phosphate characterization was performed by measuring the absorption spectra of 0.125 mM Np(V) in the mineral salts media without NTA during NaOH titration. A similar procedure was completed on a 0.125 mM Np(V)-20 mM H_3PO_4 solution for comparison. $\text{NpO}_2\text{PO}_4^{2-}$ and $\text{NpO}_2\text{HPO}_4^-$ species were characterized by comparison with their known absorption bands (22).

Samples for NTA concentration analysis were prepared by adding 0.2 ml of 0.2- μ m filtered solution to 1 ml of 10^{-3} M HCl. NTA analyses were performed with a Dionex DX-500 Ion Chromatograph (IC). The IC was calibrated using freshly prepared NTA standards. NTA

124 peak location was verified after sample analysis by spiking one sample with additional NTA and
125 re-analyzing.

126 RESULTS AND DISCUSSION

127 Neptunium toxicity and stability towards *Chelatobacter heintzii* in glucose growth media.

128 Neptunium-glucose experiments were run at two different initial cell densities to determine if
129 potential Np(V) toxicity was related to cell concentration. Microbial growth was tracked for all
130 samples for 40 days. Figure 1a shows that NpO_2^+ concentrations of 5×10^{-5} M rapidly inhibited
131 the growth of *C. heintzii* independent of initial cell density. A smaller inhibitory effect was
132 observed from lower neptunium concentrations after 10 days. Plate counts of resting cells in the
133 presence of 2.5×10^{-4} M NpO_2^+ showed a 50% loss in viability after 1 day (data not shown), and
134 OD measurements (Figure 1b) indicated that this concentration of NpO_2^+ caused complete
135 inhibition within 200 hours. Although the exact mechanism responsible for growth inhibition
136 can not be conclusively discerned from these data, the rapid onset of growth inhibition at NpO_2^+
137 concentrations $> 5 \times 10^{-5}$ M suggests that neptunium inhibition is chemical rather than radiolytic
138 in nature, since the damage caused by ionizing radiation is a function of the absorbed radiation
139 dose, a time-dependent parameter.

140 Neptunium partitioning between aqueous and particulate phases was monitored in these
141 experiments by absorption spectrometry and alpha scintillation counting of unfiltered vs 0.2- μm -
142 filtered samples. The NpO_2^+ concentrations determined from VIS-NIR spectrometry shown in
143 Figure 2 indicate that the Np(V) concentration was stable over the course of the experiment.
144 However, scintillation counting showed that 0 to 20% of the ^{237}Np was associated with the
145 particulate phase (data not shown). Since the most significant partitioning was measured in
146 samples with high neptunium and biomass concentrations, we believe that the scintillation

partitioning results are an artifact of the filtration procedure, rather than an indication of significant biosorption. We would not expect biosorption to be a significant mechanism in the Np(V) system, since NpO_2^+ sorption on organic surfaces is much less significant than that of Np(IV), due to the large difference in charge between the two ions (29).

Stability of the Np-NTA complex during NTA degradation. The growth rate of *C. heintzii* in NTA mineral media could be expected to be adversely affected by the presence of neptunium in two ways. Because NTA is a strong chelator, most neptunium in solution will form a neptunium-NTA complex, effectively rendering a portion of the NTA unavailable for biological utilization. However, chemical equilibrium speciation calculations predict that even the highest concentration neptunium sample could only complex with $\approx 2\%$ of the initial NTA in solution. Thus, the concentration-dependent growth rate of *C. heintzii* should remain unaffected by the amount of neptunium present in these experiments, as long as sufficient excess NTA is present.

The second possible mechanism that could affect the biological growth rate is the potential toxicity of neptunium towards *C. heintzii*, either as a result of metal toxicity (free ion) or due to radiolytic (free or complexed neptunium) effects. Results from the neptunium-glucose experiments showed that significant metal toxicity would not be expected unless the concentration of the free Np(V) released from the NTA complex exceeded 5×10^{-5} M. In order to isolate potential radiotoxicity effects from neptunium chemical toxicity, the minimum starting NTA:neptunium ratio for these experiments was 50:1. Chemical speciation calculations indicated that the highest free NpO_2^+ concentration was 1.3×10^{-5} M under the initial conditions; therefore, the chemical toxicity effects from the free ion should have been small.

Figure 3 shows the growth of *C. heintzii* in NTA mineral media in the presence of increasing neptunium concentrations. Growth appeared to be unaffected by neptunium

170 concentrations up to 10^{-4} M, a result verified by viable plate counts taken during the course of the
171 experiments: Culture plates prepared after 45 days showed that cells grown in the presence of
172 10^{-4} M neptunium had similar colony numbers to the control samples, suggesting that
173 radiotoxicity was not important at this concentration. The dose-to-solution at the highest
174 neptunium concentration is well below the lethal external radiation dose for *C. heintzii*, 330 Gy
175 (33 Krad) (26): At a neptunium concentration of 1.25×10^{-4} M, the effective ionizing radiation
176 dose is ≈ 0.05 Gy (5 rad) per day, or approximately 2.5 Gy (0.25 Krad) over the course of the 48
177 day experiment.

178 Previous work by Reed (26) in the Pu(IV)-NTA system showed that radiolytic effects, due
179 to preferential association of Pu(IV) with the biomass, were observed at radiation doses below
180 the lethal dose. Figure 4 shows the amount of neptunium associated with *C. heintzii*, as
181 determined by α -particle scintillation counting of 0.2- μ m filtered vs. unfiltered samples.
182 Partitioning to the solid phase was negligible for the first 250 hours, but then rapidly increased.
183 Figure 5 illustrates that, at the end of the experiment, the amount of neptunium in the filtrate was
184 roughly proportional to the total neptunium concentration in solution.

185 Although Pu(IV) associated with *C. heintzii* cells was not readily extractable with EDTA
186 (26), 0.001 M NTA recovered 97% of the neptunium from the particulate fraction, verifying that
187 the particulate neptunium was nearly completely recoverable. Approximately 41% of the
188 particle-associated neptunium was extracted by 10-fold dilution with deionized water of one of
189 the 10^{-4} M neptunium samples. The resulting particulate neptunium was 55%, which
190 corresponds with the measured particulate neptunium in the 10^{-5} M neptunium sample.
191 Therefore, the neptunium partitioning was reversible and concentration dependent within the

short time frame of the extraction procedure, indicating that the free Np(V) ion concentration was reaching chemical equilibrium with the particulate fraction.

Neptunium Speciation During Glucose and NTA Biodegradation. Reversible, concentration-dependent partitioning and lack of microbial growth inhibition suggest that neptunium released from the NTA complex was being scavenged by other ligands in the system, making it unavailable to *C. heintzii*. Subsequent colloid formation or precipitation would explain neptunium association with the biomass. In order to determine the mechanism responsible for mitigating the potential toxicity of neptunium released from NTA complexation, chemical equilibrium modeling was combined with spectrophotometric investigations to establish the changing chemical speciation of neptunium in the growth media during NTA degradation.

VIS-NIR spectrometry during the glucose media experiments verified that the Np(V) oxidation state is stable at neutral pH in the presence of *C. heintzii*. The stability of the Np(V)-NTA complex was monitored by periodically measuring the absorption spectrum during NTA degradation by *C. heintzii*. Figure 6 compares spectra from a 1.25×10^{-4} M neptunium-NTA sample after 2 hours and 3, 4, 6 and 48 days of growth. The complex was initially stable in the presence of biological activity for up to 6 days. Loss of the neptunium-NTA complex from solution at 48 days is reflected by the disappearance of the absorption band at 990.2 nm.

Two possible explanations can account for the association of neptunium with the biomass following NTA degradation by *C. heintzii*. First, free NpO_2^+ may have been adsorbed onto the cell membrane, as is seen in the Pu system. However, results from glucose-media experiments suggested that biosorption of neptunium is not a significant process. An alternative explanation is that neptunium released from the NTA complex could have formed a solid phase if NTA degradation by *C. heintzii* changed the solution characteristics such that neptunium precipitation

215 became favorable. Figure 7 shows an enlargement of the 48 day spectrum of the unfiltered 10^{-4}
216 M neptunium sample shown in Figure 6. The unfiltered spectrum shows a new band formation
217 at ≈ 1015 nm, which is indicative of the formation of a new neptunium species.

218 Precipitate formation should be correlated with the loss of NTA from solution, since
219 excess NTA would lead to a stable NTA complex. The small 990-nm peak for a filtered sample,
220 shown in the inset of Figure 7, corresponded with the final concentration of NTA at the end of
221 the experiment, shown in Table 2, and the maximum amount of neptunium that could be
222 complexed by NTA. All samples showed nearly complete NTA degradation, verifying that
223 neptunium speciation was no longer being dominated by NTA complexation.

224 The 1015 nm peak observed in Figures 6 and 7 has not been previously reported for
225 Np(V) VIS-NIR absorption spectra. However, carbonate and phosphate complexation with
226 Np(V) are known to cause red-shifted new band formation in the VIS-NIR. Carbonate is present
227 in groundwater as a byproduct of organic degradation and as a result of mineral dissolution, and
228 is a strong actinide complexant that can affect actinide mobility in subsurface aquifers (5).
229 Neptunium solubility in many carbonate systems is controlled by a $\text{Me(I)NpO}_2\text{CO}_3$ precipitate,
230 where Me(I) represents any mono-valent cation. Using a representative solubility product of
231 $10^{-10.28}$ (24), the expected NpO_2^+ concentration in equilibrium with a $\text{Me(I)NpO}_2\text{CO}_3$ solid, 0.1 M
232 Me(I), and atmospheric $\text{CO}_2(\text{g})$ at pH 8, the highest final pH of the growth media after NTA
233 degradation, would be $\approx 2.6 \times 10^{-4}$ M. All the final measured NpO_2^+ concentrations shown in
234 Table 2 were below this level, suggesting that neptunium solubility was being controlled by
235 another solid phase.

236 Chemical equilibrium calculations by Morgenstern and Kim (22) have shown that
237 phosphate complexation with neptunium can out-compete carbonate complexation in natural

waters when phosphate concentrations exceed 10^{-4} M. Although phosphate solid phases have been characterized for some actinide systems, no solubility data are available for the Np(V)-phosphate system.

To predict the dominant neptunium complexes in the system, we used the computer program CCBATCH to simulate the equilibrium chemical speciation of the mineral growth media during NTA degradation by *C. heintzii*. The aerobic biodegradation of NTA can be described by the following stoichiometry when HNTA^{2-} is selected as the degradable form of NTA (3, 28):

where $\text{C}_5\text{H}_7\text{O}_2\text{N}$ represents biomass.

Inspection of the reaction stoichiometry explains the pH rise observed in weakly buffered systems undergoing NTA degradation. Each mole of HNTA^{2-} degraded consumes 2.4 acid equivalents, causing the rise in pH. The magnitude of this effect depends on the concentration of other buffers in the system and may also be mitigated by retention in solution of a portion of the 3.4 moles of carbonic acid produced per mole of NTA degraded. For example, in a closed system, carbonic acid retention causes a less dramatic pH increase as compared to a solution in equilibrium with atmospheric $\text{CO}_2(\text{g})$. Additionally, precipitation of $\text{Np}-\text{CO}_3^{2-}$ or $-\text{PO}_4^{2-}$ solids would remove base equivalents from the system at near neutral pH, which could also counter increasing pH.

The final pHs for all the neptunium-NTA samples are shown in Table 2. As predicted by the degradation stoichiometry, consumption of NTA caused a 0.6 to 1 pH unit increase. Modeling studies of the growth medium speciation in equilibrium with atmospheric $\text{CO}_2(\text{g})$

partial pressures ($10^{-3.5}$ atm) yielded a final pH prediction of 8.5, indicating that pH was being affected by carbonic acid retention and/or precipitation of base equivalents. Figure 8 shows the predicted dominant speciation of the 0.125 mM Np(V)-NTA mineral media solution in equilibrium with a slightly elevated $\text{CO}_2(\text{g})$ partial pressure of $10^{-2.7}$ atm, which yielded a final predicted pH of 7.9. The modeling results show that $\text{NpO}_2\text{HPO}_4^-$ and $\text{NpO}_2\text{PO}_4^{2-}$ are the dominant neptunium species after significant NTA degradation has taken place. Additionally, the final NpO_2^+ ion concentration calculated by the model, 1.4×10^{-5} M, exceeds the final measured concentrations in Table 2, indicating that solubility of a neptunium solid phase is limiting the amount of neptunium in solution.

Figure 9 shows the model predicted distribution of neptunium among the major complexing ligands during NTA degradation at $10^{-2.7}$ atm $\text{CO}_2(\text{g})$ partial pressure. It illustrates that phosphate complexation accounts for 80% of the neptunium speciation in solution after complete NTA degradation. Consequently, we attempted to experimentally correlate the 1015 nm peak formation with the phosphate system. In order to identify the 1015 nm peak, the variable-pH VIS-NIR spectra of Np(V) in the defined mineral salts media (without any NTA) was compared to the Np(V) spectra obtained in a 20 mM H_3PO_4 solution. The behavior of both systems followed the results seen by Morgenstern and Kim (22) in lower concentration phosphate solutions: Figure 10 shows that as pH was increased, neptunium speciation shifted from the free aquo species to successive complexation as NpHPO_4^- (≈ 988 nm) and NpPO_4^{2-} (≈ 993 nm). Figure 11 shows that, after ≈ 20 minutes, new band formation at 1015 nm was observed at the expense of the NpPO_4^{2-} complex, and the overall turbidity of both solutions increased, suggesting colloid formation/precipitation. The final spectrum remained stable for days, (Figure 12), but the colloidal species had to be re-suspended in solution prior to each spectroscopic analysis. These

283 results indicate that neptunium speciation after NTA degradation was controlled by the
284 phosphate system, and neptunium-phosphate colloid formation/precipitation can be identified by
285 VIS-NIR peak formation at 1015 nm.

286 Our results correlate with work by Francis et al (11), who showed that nickel toxicity was
287 modulated by coprecipitation of Ni with iron-hydroxides after citrate degradation. Also,
288 formation of a neptunium-phosphate colloidal/precipitate phase agrees with results reported for
289 the Pu(VI) system (32, 33), suggesting that phosphate colloid formation/precipitation may be an
290 important mechanism affecting actinide solubility and speciation in natural systems. This latter
291 finding will be investigated further, since phosphorus is a required biological nutrient and many
292 in-situ bioremediation efforts require supplemental phosphorus additions.

293 Our results in this study are in contrast to previous findings in the plutonium-NTA
294 system, where *C. heintzii* was sensitive to radiolytic effects at similar actinide concentrations due
295 to selective association of plutonium with the biomass. This work suggests that future
296 bioremediation efforts involving actinide elements will require careful evaluation of coupled
297 chemical and biological processes.

298 CONCLUSIONS

299 Toxicity studies in non-complexing glucose growth media showed that aquo NpO_2^+ is
300 stable in the presence of aerobic biological activity for up to 40 days. The uncomplexed Np(V)
301 ion significantly inhibited growth of *Chelatobacter heintzii* at concentrations greater than or
302 equal to 5×10^{-5} M, most likely due to toxic metal inhibition.

303 Our results show that growth of *C. heintzii* in NTA media is unaffected by the presence of
304 up to 10^{-4} M Np(V) in solution after 48 days. Viability of *C. heintzii* remains unaffected by the
305 presence of 10^{-4} M neptunium after complete degradation of all NTA in solution.

306 Dynamic chemical equilibrium modeling, combined with VIS-NIR absorption
307 spectrometry data, suggest that the potential toxicity of neptunium to *C. heintzii* is mitigated by
308 neptunium-phosphate precipitation induced by a pH increase caused by biodegradation of NTA.

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312 directed research funds to investigate actinide speciation in environmental systems. The
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APPENDIX A

CCBATCH modeling methodology and application to the neptunium-NTA system.

CCBATCH is a computer code, developed at Northwestern University, designed to investigate the dynamics of coupled biological and chemical reactions in mixed waste subsurface environments. The exact modeling methodology is describe elsewhere (28). To summarize, the model is unique in that it explicitly couples biological electron donor and acceptor substrate consumption to competing chemical reactions in order to determine the effect of biological reactions on the fate of various components in the system. Currently, the model systematically considers the following competing subsurface reactions: acid/base, complexation, fixed-pH precipitation, biomass synthesis and decay, substrate utilization (contaminant degradation), and toxicity effects.

Based on user input information, the model calculates the equilibrium concentrations of all the chemical species in the system, including the degradable form of the electron donor substrate, at the initial pH and component concentrations. It uses a Newton-Raphson technique that combines the aqueous-phase mass balances with mass-action equilibrium expressions for all relevant acid/base and complexation reactions, and can it compute the equilibrium pH from the proton condition when the pH is not fixed.

Once the initial acid-base and complexation equilibria are established, the model uses the method developed by McCarty (19) to calculate the fraction of available electrons (received from the electron donor) utilized in energy generating, biomass production, and biomass decay reactions. By determining the proper rationing of electrons between these various pathways, the substrate utilization and biomass synthesis stoichiometry are calculated based on the degradable form of substrate input to the model. The correct coupling of substrate utilization to acidic

hydrogen production or consumption is the important link that enables the model to calculate changes in system pH caused by substrate consumption.

Once the pH change and component total concentration adjustments are determined for a given time step, the model recalculates the acid/base and metal complex speciation of the system based on the updated component concentrations and new pH. The newly calculated substrate concentrations are used to determine the substrate utilization rate for the next time step, and the process is repeated until the limiting substrate is exhausted. The model allows prediction of substrate utilization rates based on the concentration of individual substrate species, as opposed to overall substrate concentrations.

To illustrate the methodology used by CCBATCH, consider the fate of neptunium during the degradation of NTA, based on HNTA²⁻ degradation stoichiometry. The rate of change of major components in the system can be related to the rate of substrate consumption as follows:

$$\begin{aligned}
 r_{\text{HNTA}} &= -q_{\text{md}} \frac{S_{\text{HNTA}}}{K_{\text{HNTA}} + S_{\text{HNTA}}} \frac{S_{\text{O}_2}}{K_{\text{O}_2} + S_{\text{O}_2}} X && \text{rate of donor utilization} \\
 r_{\text{net}} &= r_{\text{syn}} + r_{\text{decay}} = -Yr_{\text{HNTA}} - bX && \text{rate of biomass accumulation} \\
 r_{\text{O}_2} &= 1.59r_{\text{HNTA}} + 5bX && \text{rate of oxygen utilization} \\
 r_{\text{H}^+} &= 2.42r_{\text{HNTA}} + 1bX && \text{rate of acidic hydrogen utilization} \\
 r_{\text{H}_2\text{CO}_3^*} &= 3.09r_{\text{HNTA}} + 5bX && \text{rate of H}_2\text{CO}_3^* \text{ production} \\
 r_{\text{NH}_4^+} &= 0.42r_{\text{HNTA}} + 1bX && \text{rate of NH}_4^+ \text{ production}
 \end{aligned}$$

where q_{md} is the maximum specific utilization rate for the electron donor substrate; K_{HNTA} is the half maximum rate concentration for the specific donor species; S_{HNTA} is the electron donor substrate concentration; K_{O_2} is the half maximum concentration for the electron acceptor, oxygen; S_{O_2} is the oxygen concentration; X is the concentration of active biomass degrading the primary substrate; Y is the true yield coefficient; and b is the biomass decay constant. The first

coefficient in each of the last four rate equations is calculated directly from HNTA^{2-} degradation stoichiometry. For example, the rate of oxygen utilization is $0.0875/0.055 = 1.59$ moles of O_2 consumed per mole of HNTA^{2-} degraded. Similarly, the second coefficient can be derived from stoichiometry representing the complete endogenous mineralization of cell biomass (28). Thus, mineralization of one mole of biomass ($\text{C}_5\text{H}_7\text{O}_2\text{N}$) consumes 5 moles of O_2 and 1 mole of H^+ , and produces 5 and 1 mole(s) of carbonic acid and NH_4^+ , respectively.

As HNTA^{2-} and acidic hydrogen are removed from the system, H_2CO_3^* and NH_4^+ are produced. To maintain chemical equilibrium, the remaining NTA re-equilibrates among its various acid-base and complex species based on the new pH and component totals. Initially, the presence of excess NTA in the system dominates neptunium speciation. However, eventually enough HNTA^{2-} is degraded that the total amount of NTA remaining in solution is no longer sufficient to complex most of the neptunium. The fate of the "free" neptunium then depends on the pH and presence of other ligands in solution.

In order to calculate the dominant neptunium speciation in the NTA growth medium, we considered the formation of 47 complexes from 16 components. The important neptunium aqueous species and their formation constants are shown in Table A-1. Formation constants for non-actinide metal complexes and acid dissociation constants were taken from Morel and Hering (21) and corrected to 0.1 M ionic strength. Carbonate speciation was calculated by assuming Henry's Law equilibrium between the aqueous and gas phase CO_2 concentrations.

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TABLE 1. Initial cell inoculations and total neptunium concentrations for
neptunium-glucose and neptunium-NTA studies^a

Experimental Code	Total Neptunium (M)	Initial Cell Concentration OD ₆₀₀
1-B-1	0	0.004
1-B-2	0	0.004
1-B-3	0	0.004
1-9-1	2.0×10^{-8}	0.004
1-8-1	1.7×10^{-8}	0.004
1-7-1	2.0×10^{-7}	0.004
1-6-1	1.7×10^{-6}	0.004
1-5-1	1.9×10^{-5}	0.004
1-4-1	1.2×10^{-4}	0.004
1-4-2	1.2×10^{-4}	0.004
2-B-1	0	0.007
2-B-2	0	0.007
2-B-3	0	0.059
2-B-4	0	0.007
2-B-5	0	0.059
2-4-1	5.4×10^{-5}	0.007
2-4-2	5.4×10^{-5}	0.007
2-4-3	5.4×10^{-5}	0.059
2-4-4	2.9×10^{-4}	0.007
2-4-5	2.7×10^{-4}	0.059
2-4-B	5.4×10^{-5}	0.0
2-6-1	5.9×10^{-6}	0.007
2-8-1	6.0×10^{-8}	0.007

^aLabels starting with 1 denote NTA mineral media. Label 2 represents minimal
glucose media. Initial NTA and glucose concentrations were 5.2 mM (0.375 gm
C/liter).

TABLE 2. Final NTA and neptunium concentrations of filtered NTA
growth medium samples after 48 days^a

Sample	Final Np(V) Concentration (M)	Final NTA Concentration (M)	Final pH
1-4-1	3.84×10^{-6}	1.04×10^{-6}	$7.90 \pm .03$
1-4-2	6.37×10^{-6}	2.91×10^{-6}	$7.98 \pm .04$
1-5-1	8.27×10^{-6}	ND ^b	$7.83 \pm .03$
1-6-1	7.68×10^{-7}	ND	$7.87 \pm .04$
1-7-1	1.52×10^{-7}	ND	$7.73 \pm .03$
1-8-1	1.7×10^{-8}	7.97×10^{-7}	$7.63 \pm .03$
1-9-1	2.0×10^{-9}	ND	$7.84 \pm .02$

^aNTA concentrations determined by IC analysis. Neptunium determined by alpha particle
scintillation counting.

^bND - Not Detected ($< 1 \times 10^{-7}$ M)

459 TABLE A-1. Equilibrium formation constants for major neptunium
460 aqueous species in NTA mineral growth medium

Species	β_{xyz}^a	References
NpO_2OH	$\beta_{1(-1)0} = 10^{-11.3}$	(12, 23)
$\text{NpO}_2(\text{OH})_2^-$	$\beta_{1(-2)0} = 10^{-23.43}$	(12, 23)
$\text{NpO}_2\text{CO}_3^-$	$\beta_{101} = 10^{4.38}$	(2, 5, 24)
$\text{NpO}_2(\text{CO}_3)_2^{3-}$	$\beta_{102} = 10^{6.55}$	(2, 5, 24)
$\text{NpO}_2(\text{CO}_3)_3^{5-}$	$\beta_{103} = 10^{6.4}$	(2, 5, 24)
$\text{NpO}_2\text{NTA}^{2-}$	$\beta_{101} = 10^{6.80}$	(7, 25)
$\text{NpO}_2\text{HNTA}^-$	$\beta_{111} = 10^{1.77}$	(7)
$\text{NpO}_2(\text{OH})\text{NTA}^{2-}$	$\beta_{1(-1)1} = 10^{-4.66}$	(7)
$\text{NpO}_2\text{HPO}_4^-$	$\beta_{111} = 10^{14.23}$	(22)
$\text{NpO}_2\text{PO}_4^{2-}$	$\beta_{101} = 10^{6.33}$	(22)

461 ^aWhere $\beta_{xyz} = \frac{[\text{M}_x\text{H}_y\text{L}_z]}{[\text{M}]^x[\text{H}]^y[\text{L}]^z}$ or $\beta_{x(-y)z} = \frac{[\text{M}_x(\text{OH})_y\text{L}_z][\text{H}]^y}{[\text{M}]^x[\text{L}]^z}$.

FIG. 1. Growth of *Chelatobacter heintzii* in glucose media (OD_{600}) as a function of time for growing (a) and resting (b) cells.

FIG. 2. NpO_2^+ concentration, determined by VIS-NIR absorption spectrometry, as a function of time ($\epsilon = 392$ @ 980.2 nm). The free ion concentration remained stable over the course of the experiment, indicating no change in oxidation state or complexation occurred during glucose degradation by *C. heintzii*.

FIG. 3. Growth of *C. heintzii* vs time in NTA mineral media. Error bars are 99% confidence limits of the mean of three blank samples.

FIG. 4. Neptunium partitioning during NTA degradation by *C. heintzii*. Partitioning was determined by alpha particle scintillation count comparison of filtered to unfiltered samples.

FIG. 5. Percent particulate neptunium as a function of total neptunium concentration at the end of the experiment. Dashed lines represent 95% confidence interval. $Np(V)$ partitioning with the solid phase was proportional to the total neptunium concentration.

FIG. 6. VIS-NIR spectra of 1.25×10^{-4} M neptunium in NTA mineral media during NTA degradation by *C. heintzii*, showing both the stability of the neptunium-NTA complex (990.2 nm peak), and microbial growth (OD_{600}). After NTA degradation, neptunium speciation shifted to the phosphate system, indicated by the 1015 nm absorption band.

FIG. 7. Enlargement of the 970 to 1050 nm region of the unfiltered 48 day spectrum shown in FIG. 6. The 1015 nm peak is clearly visible. A small amount of neptunium-NTA complex was seen after filtration (990 nm peak, inset).

FIG. 8. Neptunium speciation during NTA degradation for 0.125 mM Np(V) in NTA mineral growth media, calculated by the dynamic chemical equilibrium computer code, CCBATCH. Calculations are based on equilibrium with $10^{-2.7}$ atm $\text{CO}_2(\text{g})$ partial pressure and the assumption that no solid phases are present.

FIG. 9. Calculated dominant neptunium complexes in the mineral growth medium as a function of amount of NTA degraded, under the same conditions noted in Figure 8. $\text{NpO}_2\text{HPO}_4^-$ and $\text{NpO}_2\text{PO}_4^{2-}$ are the dominant neptunium species following NTA degradation, accounting for 80% of the total neptunium. The $\text{NpO}_2\text{CO}_3^-$ species accounted for less than 10% of the total neptunium in solution after NTA degradation.

FIG. 10. VIS-NIR absorption spectra of 0.125 mM Np(V) in NTA-free mineral salts growth media at various pH. The dominant neptunium species in solution are NpO_2^+ (980.2 nm (10)), $\text{NpO}_2\text{HPO}_4^-$ (988.8 nm (22)), and $\text{NpO}_2\text{PO}_4^{2-}$ (993.6 nm (22)). Spectra matched those obtained for a 20 mM H_3PO_4 solution (not shown).

FIG. 11. VIS-NIR absorption spectra of 0.125 mM Np(V) in NTA-free mineral salts growth media at pH 7.87 (a), and in a 20 mM H_3PO_4 solution at pH 8.22 (b), both as a function of time. Predominant neptunium speciation shifts from phosphate complexation to an unidentified colloidal/precipitate phase (1015 nm). Note the increase in solution turbidity (baseline OD_{960} shift from 0.011 to 0.031) caused by agitation.

501 **FIG. 12. VIS-NIR absorption spectra of 0.125 mM Np(V) in NTA-free mineral salts**
502 **growth media at pH 7.87 after 3 days (a), and in a 20 mM H₃PO₄ solution at pH 8.22 after**
503 **1 day (b). The neptunium-phosphate colloidal/precipitate phase (1015 nm) remained**
504 **stable. Once again, agitation increased the turbidity of the solution.**

Figure 1 - Growth of *Chelatobacter heintzii* in glucose media (OD_{600}) as a function of time for growing (a) and resting (b) cells.

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