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Radiotoxicity of plutonium in NTA-degrading *Chelatobacter heintzii* cell suspensions *

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Abstract

The radiotoxicity of plutonium in NTA-degrading *Chelatobacter heintzii* cell suspensions was investigated as part of a more general study to establish the key interactions between actinide-organic complexes and microorganisms in the subsurface. The radiation tolerance of *C. heintzii*, based on ^{60}Co gamma irradiation experiments, was 165 ± 30 Gy. No bacteria survived irradiation doses greater than 500 Gy. In the presence of plutonium, where alpha particle decay was the primary source of ionizing radiation, the observed toxicity was predominantly radiolytic rather than chemical. This was evident by the greater effect of activity, rather than concentration, on the toxicity noted. Bioassociation of plutonium with *C. heintzii* was postulated to be an important and necessary step in the observed loss of cell viability since this was the best way to account for the observed death rate. The radiotoxicity of plutonium towards bacteria is a potentially important consideration in the bioremediation of sites contaminated with radionuclide-organic mixtures and the bioprocessing of nuclear waste.

Abbreviations: DOE – U. S. Department of Energy, HPW – High purity water, NTA – Nitrilotriacetic acid, PIPES – piperazinebisethanesulfonic acid buffer, LET – linear energy transfer of ionizing radiation in matter

Introduction

The effects of microbial activity on the migration and immobilization of radionuclides are potentially important considerations in the bioremediation of sites contaminated with radionuclide-organic waste mixtures (Banaszak et al., 1998; Riley and Zachara, 1992; Silva and Nitsche, 1995; Liese, 1996). The radiotoxicity of actinides may limit the extent of biodegradation hence the overall utility of bioremediation to clean up contaminant mixtures in the subsurface.

That ionizing radiation is detrimental to cell viability is well-established (Ewing, 1987; Johansen and Howard-Flanders, 1965; Ewing, 1973; Ewing, 1982a; Ewing, 1982b). These studies are focused on the use of radiation therapy to treat cancer and emphasize radiation damage to eukaryotic cells. Cell damage and death is explained by the reactions of oxidizing free radicals (e.g., $OH^{\cdot}$ and $HO_2^{\cdot}$) and molecular products (hydrogen peroxide) generated both within and outside of the cell in aqueous media by ionizing radiation. These radiolytic species react with key proteins and nucleic acids in the cell leading to the breakdown of DNA strands, the disruption of cell metabolism, and consequently the loss of cell viability (i.e., ability to reproduce).

Ionizing radiation, rather than chemical toxicity, was hypothesized to be the predominant cause of cell death when bacteria isolated from subsurface environments interact with plutonium (Wildung et al., 1987; Wildung and Garland, 1980; Wildung and Garland, 1982). In the presence of plutonium, the source of ionizing radiation is primarily the alpha particle decay of the actinide. When the cells are suspended in an

aqueous medium (e.g., groundwater), the energy deposition occurs in both the medium and the cell, leading to dose-to-solution and dose-to-cell contributions to the loss of cell viability.

In the work reported herein, the radiotoxicity of plutonium towards suspensions of *Chelatobacter heintzii* was established. In the absence of complexation, Pu(IV) has a strong tendency to hydrolyze/polymerize at pH > 4. For this reason, the Pu(IV) was initially present as an organic complex. These complexes are of interest in the subsurface because of their potentially high mobility. The aerobic degradation of NTA by *C. heintzii* is a relatively well-understood system (Bolton et al., 1996; Egli, 1994; Reed et al., unpublished) that is being used to investigate plutonium-microbiological interactions under groundwater relevant conditions. The biodegradation of NTA by *C. heintzii* is described by the following reaction (Rittmann and VanBriesen, 1996):

$$0.055\ C_6H_7O_2N^{2-} + 0.0875\ O_2 + 0.133\ H^+ + 0.069\ H_2O \rightarrow 0.032\ C_5H_7O_2N\ \text{(biomass)} + 0.17\ H_2CO_3 + 0.023\ NH_4^+$$

Net degradation products are carbon dioxide and ammonia.

In our studies, the biodegradation of NTA destabilized the plutonium-NTA complex leading to the bioassociation of plutonium with *C. heintzii*. Initially, the alpha particles were primarily deposited in solution (from the Pu(IV)-NTA complex). After bioassociation, the alpha particles were primarily deposited in the cellular material. The radiation tolerance of the bacteria was also determined by performing gamma irradiation studies to help establish the extent that alpha particle deposition in solution contributed to the overall loss of cell viability. Different isotopes of plutonium were used as the radiation source to separate chemical and radiolytic effects. This investigation is part of a more general study to establish actinide speciation in the subsurface and identify key subsurface interactions between actinide-organic complexes and bacteria that lead to the mobilization and immobilization of actinides (Reed et al, 1995; Reed et al., 1992; Beitz et al., 1988; Banaszak and Reed, 1997; Banaszak et al., 1998; Banaszak et al., accepted).

Materials and methods

Two isotopes of plutonium were used to vary the radioactivity of the actinide: ^{239}Pu (>99% purity by mass and activity) and ^{242}Pu (>99% isotopic purity by mass, 50% purity by activity). Reagent grade nitrilotriacetic acid (NTA), >99% purity from Mallinckrodt, was used without further purification. The pH buffer 1,4-piperazinebisethanesulfonic acid (PIPES), >99% purity from Mallinckrodt, was used as received without further purification. High-purity water (>18 MΩ nanopure-filtered water designated HPW) was used in all the experiments performed.

The bacterium used in these studies was *Chelatobacter heintzii* (ATCC-29600), which is a well-characterized NTA degrader (Bolton et al., 1996; Egli, 1994). *C. heintzii* was grown in a controlled temperature shaker at 30 °C in air-saturated NTA growth medium (Bolton et al., 1996) comprised of 1.6 g/L K_2HPO_4, 0.4 g/L KH_2PO_4, 0.5 g/L ammonium nitrate, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 25 mg/L $CaCl_2 \cdot 2H_2O$, and 2.5 mg/L $FeCl_3 \cdot 6H_2O$. Cells were grown to an optical density (OD) of ~0.1 to 0.2 at 600 nm corresponding to a viable cell density of ~3–7 × 10^8 cells/mL. The bacteria were harvested in log growth phase immediately prior to use. The cells were concentrated by centrifugation and washed twice with sterile 0.01 M PIPES solution to remove residual NTA and establish the desired cell concentration (typically OD_{600} = 0.4–0.6).

The Pu solution was taken to dryness, dissolved in 8 M nitric acid to remove organics, and passed through a bio-rad ion-exchange column to remove inorganic ions present. The synthesis of the plutonium(IV)-NTA complex was complicated by the tendency of Pu(IV) to hydrolyze and form insoluble polymers at pH >4. To circumvent this problem, the plutonium stock was first reduced to Pu(III) by taking to dryness in hydrobromic acid (48% solution), and then re-dissolved in 0.01 M PIPES buffer containing the desired concentration of NTA. The dissolved-air oxidation of Pu(III) to Pu(IV) resulted in the preferential formation of the Pu(IV)-NTA complex. The formation of plutonium polymers was negligible because the effective concentration of uncomplexed Pu(IV) remained low. As a result of this approach, some bromide was left in solution. We showed, in supporting experiments, that the levels of bromide present did not affect the viability of the microbe in our system. Absorption spectra of the plutonium complex, shown in Figure 1, were taken to verify its speciation as a Pu(IV)-NTA complex.

The viability of *C. heintzii* was established by periodically removing the irradiated samples and performing bioassays. Unirradiated samples were analyzed at the same point in time to account for natural cell

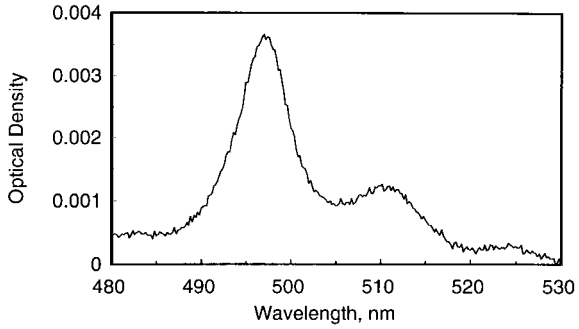

Figure 1. Absorption spectrum of the 10^{-5} M Pu(IV)-NTA complex in PIPES buffer at pH 6.2.

death. The bioassays consisted of plating serial dilutions of the cell suspension on agar culture plates made with the NTA growth medium. Cell viability was established by counting the number of colony-forming units (cfu) formed. This corresponds to about 60% of the viable cells actually in the suspension based on comparisons of our cfu data to direct cell counts using a microscope. There was usually a lag-time in the growth of colonies on the plates, typically 1–2 weeks, for the cell suspensions that had been exposed to ionizing radiation (both gamma and alpha). For this reason, the cfu on each plate were counted over a period of six weeks, until no further increase in the number of cfu was observed.

The concentration of NTA was measured using a Dionex DX-500 ion chromatograph with an AS-11 column in isocratic mode at an eluent strength of 20-mM sodium hydroxide. The partitioning of plutonium between the biomass, which may include bio-associated plutonium species, and aqueous phase was established by alpha scintillation counting (Packard model 2500 TR liquid scintillation analyzer) of both the 0.2-μm-filtered and unfiltered fractions of each sample. Selected samples were also analyzed spectroscopically to establish/monitor the speciation of plutonium in solution with a Varian CARY-5 spectrophotometer.

Plutonium-NTA-C. heintzii experiments

The plutonium-NTA-*C. heintzii* studies were performed using the same experimental procedure for both plutonium isotopes. The desired concentration of plutonium (10^{-5} M to 10^{-7} M) in 0.01 M PIPES with 0.2 mM NTA at pH = 6.2 $\pm$ 0.1 was prepared by adding the appropriate amount of the Pu(IV)-NTA stock solution to 20–30 mL of NTA-PIPES solution. The stability of the plutonium-NTA complex, in the absence of bacteria, was established at the higher plutonium concentrations by monitoring the absorption spectrum as a function of time. At lower plutonium concentrations, [Pu] $< 10^{-6}$ M, the stability of the complex was determined indirectly by alpha scintillation counting after filtration.

Experiments were initiated by inoculating the plutonium-NTA-PIPES solution (minimal media as described in Bolton et al., 1996) with 1 mL of the concentrated cell suspension. The initial cell density of *C. heintzii* was $6.0 \pm 1.5 \times 10^7$ cells/mL, corresponding to an OD of 0.018 @ 600 nm and a biomass dry weight of 12 mg/L. The experiments were conducted in un-stirred glass bottles, at a pH of 6.2 $\pm$ 0.1, at room temperature (21 $\pm$ 2 °C), in the presence of air, and in the dark. Sterile pipettes (autoclaved) were used for solution transfers, and all solutions were filter-sterilized (0.2 μm filter) prior to use. Serum bottles and glassware used were autoclaved prior to their use. The bottles used were capped during the experiments performed. Time-dependent dissolved oxygen measurements showed that oxygen depletion was not a factor in the experimental method we used. Supporting studies also showed that there was no difference in the rate of NTA degradation when static experiments were compared to those that were shaken continuously. There was no evidence of bacterial contamination in any of the experiments performed. Cell growth during the two-week experiments was negligible (i.e., less than 10%).

Gamma irradiation experiments

Suspensions of *C. heintzii* in high-purity water and 0.01 M PIPES solution (pH = 6.2) were irradiated to absorbed doses of up to 30,000 Gy (3 Mrad). Gamma irradiations were performed with a ^{60}Co gamma source at dose rates of approximately 1000 Gy/h. A few of the high absorbed-dose experiments were done at 3000 Gy/h. Fricke dosimetry (Spinks and Woods, 1976) was performed to establish the gamma dose rate using 5 mL of dosimeter solution in the same-size test tubes used in the cell irradiations. This measured the gamma dose absorbed by the solution.

The PIPES solution, which was at the same PIPES concentration and pH, most closely simulated the solution environment of the plutonium-containing experiments. The cell density was also similar to that used in the plutonium-containing experiments. The irradiations were performed by inoculating the microbe into

5 mL of filter-sterilized media in a glass test tube with an aluminum foil cap (both autoclaved prior to use). These samples were irradiated at fixed locations for durations up to 20 h (typically, however, only a few minutes).

In both the HPW and PIPES solutions, no NTA was present. This eliminated the radiolysis of NTA as a potential complexity and minimized cell growth. Data were plotted in terms of the fractional survival of *C. heintzii*, defined as the number of cfu obtained for a particular irradiation experiment divided by the number of cfu measured, at the same point in time, for the unirradiated reference case. Completely analogous unirradiated experiments were performed at the same time, in triplicate, to establish the natural rate of cell decay.

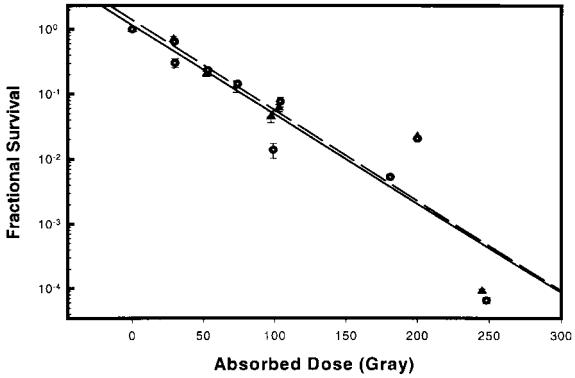

Figure 2. Effect of gamma irradiation on the viability of *C. heintzii* in HPW (●) and 0.01 M PIPES buffer solution (▲). Data for both experiments are shown together. Error bars are based on the range in cfu counts obtained for each experiment.

Results and discussion

Three series of experiments were performed to differentiate between chemical and radiolytic contributions to the radiotoxicity of plutonium toward *C. heintzii.* First, the radiation tolerance of *C. heintzii* was established in gamma-irradiation studies. Second, the survival of *C. heintzii* was established as a function of the concentration of the ^{239}Pu(IV)-NTA complex, where the source of ionizing radiation was the alpha particle decay of plutonium in a system where the distribution of plutonium was evolving. Third, completely analogous experiments were performed with the ^{242}Pu(IV)-NTA complex to vary the activity.

Gamma irradiation of C. heintzii

The experiments performed, irradiation conditions, and resulting cfu counts are given in Table 1. Two separate sets of experiments were done. These were designated CH-IRRAD-1 (replicate samples) and CH-IRRAD-2 (triplicate samples at each absorbed dose). The log (fractional survival) is linear with absorbed dose (see Figure 2) and not affected by the presence/absence of the PIPES buffer. A regression fit of the PIPES data [log(fractional survival) vs. krad] had a correlation coefficient of $r^2 = 0.96$, a y-intercept of -0.09 ± 0.09, and a slope of -0.102 ± 0.011. The radiation tolerance of *C. heintzii*, defined as the absorbed dose where only 1% of the bacteria survived, was 187 ± 13 Gy (18.7 ± 1.3 krad) based on this empirical relationship. A similar analysis of the data for experiment CH-IRRAD-2 gave a radiation tolerance of 144 ± 15 Gy (14.4 ± 1.5 krad) based on the slope. Overall, accounting for both experiment sets, the radiation tolerance measured was 165 ± 30 Gy.

Additional gamma-irradiations were conducted to absorbed doses of 500 to 30,000 Gy. In all cases, on the basis of cfu counts, no cells survived. Cells from these higher absorbed-dose irradiations (>50 Gy) were transferred to NTA growth medium and shaken at 30 °C. In all cases, these cells did not grow, indicating that the loss of cell viability at these higher absorbed doses was complete.

Gamma irradiation with a ^{60}Co source (1.33 and 1.17 MeV gamma rays) corresponds to low linear energy transfer (LET) radiation that is well within the Compton regime (i.e., energy deposition is by inelastic scattering). The radiation chemistry in aqueous media is induced by inelastic scattering of high-energy secondary electrons emitted as a result of the absorption of the gamma ray. Energy deposition is widely scattered throughout the medium and primarily deposited in the aqueous medium rather than the biomass because the volume fraction of the cells is ∼0.005% under our experimental conditions. For these reasons, we believe that the effects on cell viability noted were primarily due to reaction of transients generated in the bulk solution with cellular material.

The gamma radiation tolerance of *C. heintzii* we determined (1% survival at an absorbed dose of 165 Gy) is comparable to that typically observed for prokaryotic cells in oxygen-sensitized environments (Ewing, 1987) where the observed loss of cell viability is primarily due to the reaction of radiolytically produced transient free radicals with nucleic acid in the cell. The similar results we obtained for cells suspended in both high-purity water and PIPES solution

Table 1. Experimental conditions and results of the *C. heintzii* gamma-irradiation studies. All experiments were done, in replicate, with $\sim 10^7$ cells suspended in a 5 mL volume. HPW – experiments performed with cells suspended in high-purity water, PIPES – cell suspended in 0.01 M PIPES buffer.

Experiment designation	Dose rate (Krad/min)	Irradiation time (min)	Absorbed dose (Krad)	cfu counts cells/mL	
				Low	High
Experiments CH-IRRAD-1					
HPW-B-1,2	0	NA	0	700 000	730 000
HPW-10-1,2	0.89	11	9.74	26 000	39 000
HPW-25-1,2	0.91	27	24.5	60	70
HPW-50-1,2	0.99	50	49.5	0	0
PIPES-0-1,2	0	NA	0	670 000	710 000
PIPES-3-1,2	0.99	3	2.97	410 000	480 000
PIPES-10-1,2	0.90	11	9.9	7 000	12 000
PIPES-18-1	0.86	21	18.1	3 300	4 000
PIPES-25-1,2	0.92	27	24.8	40	50
PIPES-50-1,2	1.00	50	50.0	0	0
Experiments CH-IRRAD-2					
HPW-B-1,2,3	0	NA	0	1 120 000	1 440 000
HPW-3-1,2,3	0.95	3	2.9	800 000	1 000 000
HPW-5-1,2,3	0.87	6	5.2	236 000	288 000
HPW-7-1,2,3	0.91	8	7.3	136 000	204 000
HPW-10-1,2,3	0.86	12	10.3	68 000	88 000
HPW-20-1,2,3	1.00	20	20.0	28 000	28 800
PIPES-B-1,2,3	0	NA	0	680 000	1 840 000
PIPES-3-1,2,3	0.99	3	3.0	320 000	440 000
PIPES-5-1,2,3	0.89	6	5.3	272 000	316 000
PIPES-7-1,2,3	0.92	8	7.4	180 000	184 000
PIPES-10-1,2,3	0.87	12	10.4	80 000	112 000
PIPES-20-1,2,3	1.00	20	20.0	24 000	28 000

indicated that the PIPES buffer, and associated radiolytic decomposition products, did not affect cell viability.

Radiotoxicity of plutonium-239 toward C. heintzii

The experiments performed to establish the radiotoxicity of plutonium are summarized in Table 2. The cfu data are summarized in Table 3. In the absence of plutonium, bacteria in the NTA/PIPES solution died slowly due to endogenous decay following the depletion of NTA. On the basis of cfu counts, over 90% of the bacteria survived for one week. This decreased to about 50% survival after two weeks. The initial presence of NTA in solution was not a critical factor since the same rate of survival was observed for cells suspended in NTA-PIPES medium (i.e., exactly analogous to the plutonium-NTA experiments) and for those in the PIPES medium without NTA present. Visual examination of the cells, after two weeks, did not reveal any evidence of cell lysis.

The addition of plutonium-239, at concentrations of $9.9 \pm 0.1 \times 10^{-6}$ M, $1.3 \pm 0.1 \times 10^{-6}$ M, and $1.3 \pm 0.1 \times 10^{-7}$ M, resulted in an increased rate of cell death with increasing plutonium concentration. In these experiments, the plutonium initially existed almost exclusively as an NTA complex. This Pu(IV)-NTA complex was stable for several months in PIPES buffer solution when *C. heintzii* was not present. The biodegradation of NTA by *C. heintzii* led to the eventual destabilization of the Pu(IV)-NTA complex and subsequent polymerization of the aqueous plutonium species.

Table 2. Summary of results from the Pu-NTA-*C. heintzii* toxicity studies.

Experiment Designation	Description	Energy deposition rate (eV/mL/day)
Plutonium-239 experiments		
N7-PU5-M	Replicate 9.9×10^{-6} M Pu(IV)-NTA, 0.0003 M NTA, *C. heintzii*, in 0.01 M PIPES	2.4×10^{15} (0.38 Gy/day)
N7-PU6-M	Replicate 1.3×10^{-6} M Pu(IV)-NTA, 0.0003 M NTA, *C. heintzii*, in 0.01 M PIPES	3.1×10^{14} (0.050 Gy/day)
N7-PU7-M	Replicate 1.3×10^{-7} M Pu(IV)-NTA, 0.0003 M NTA, *C. heintzii*, in 0.01 M PIPES	3.1×10^{13} (0.0050 Gy/day)
N7-PU5,6,7-B	9.9×10^{-6}, 1.3×10^{-6} and 1.3×10^{-7} M Pu(IV)-NTA, 0.0003 M NTA, in 0.01 M PIPES with no *C. heintzii*	2.4×10^{15}, 3.1×10^{14}, 3.1×10^{13}
N7-M	Triplicate *C. heintzii* with 0.0003 M NTA in 0.01 M PIPES	0
N7-NTA	0.0003 M NTA in 0.01 M PIPES	0
N7-M-B	Replicate *C. heintzii* in 0.01 M PIPES	0
Plutonium-242 experiments		
N8-PU5 (0.047 Gy/day)	Triplicate 1.0×10^{-5} M Pu(IV)-NTA, 0.0003 M NTA, *C. heintzii*, in 0.01 M PIPES	3.0×10^{14}
N8-PU5-B	1.0×10^{-5} M Pu(IV)-NTA, 0.0003 M NTA in 0.001 M PIPES	3.0×10^{14} (0.047 Gy/day)
N8-M	Replicate *C. heintzii*, 0.0003 M NTA in 0.01 M PIPES	0
N8-B	*C. heintzii* in 0.01 M PIPES	0

Table 3. Summary of cfu data for the plutonium-NTA-*C. heintzii* experiments. Data shown (cfu) are the average cfu counts and have an uncertainty of ±30%. Fractional survival is the ratio of the cfu measured divided by the cfu in the non-plutonium experiments (N7-M and N8-M).

Time (h)	N7-PU5-M		N7-PU6-M		N7-PU7-M		N7-M	
	cfu	Fractional survival	cfu	Fractional survival	cfu	Fractional survival	cfu	Fractional survival
N7 experiment set – [Pu-239] effect on toxicity								
29.2	860 000	0.78	870 000	0.79	1 030 000	0.94	1 100 000	NA
53.7	660 000	0.74	890 000	1.0	870 000	0.98	890 000	NA
122.7	60 000	0.048	500 000	0.40	500 000	0.40	1 250 000	NA
292.2	50	4.7×10^{-5}	110 000	0.10	560 000	0.53	1 060 000	NA

Time (h)	N8-PU5-M		N8-PU6-M		N8-PU7-M		N8-M	
	cfu	Fractional survival	cfu	Fractional survival	cfu	Fractional survival	cfu	Fractional survival
N8 experiment set – toxicity at [Pu-242] $\sim 1 \times 10^{-5}$ M								
6.2	1 800 000	0.82	1 600 000	0.73	1 700 000	0.77	2 200 000	NA
25.6	1 700 000	0.77	1 700 000	0.77	1 600 000	0.73	2 200 000	NA
97	1 000 000	0.4	1 200 000	0.48	1 000 000	0.4	2 600 000	NA
173	320 000	0.15	450 000	0.20	350 000	0.16	2 200 000	NA

NA – not applicable.

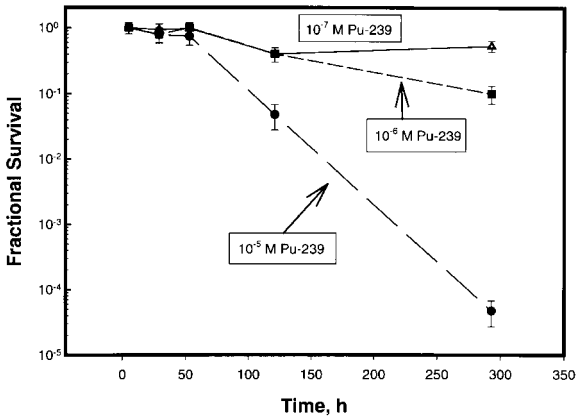

Figure 3. The fractional survival of *C. heintzii* as a function of time and concentration of the Pu(IV)-NTA complex. Data shown are based on cfu counts.

The fractional survival of *C. heintzii* during NTA degradation in the presence of plutonium-239 is shown in Figure 3. The presence of plutonium, up to concentrations of $\sim 10^{-6}$ M, did not cause significant loss in cell viability during the first 50 h. Plutonium concentrations of $\sim 10^{-5}$ M caused a small but significant decrease in cell viability, corresponding to a fractional survival of 0.74.

Significant radiotoxicity effects were, however, evident after five days (based on cfu counts). These effects increased nonlinearly with increasing plutonium concentration. For the two lower plutonium concentrations, the fractional survival was 0.4. At the highest plutonium concentration of $\sim 10^{-5}$ M, the fractional survival was 0.048, which is a factor of 8 lower. After 12 days, fractional survival was 0.5, 0.1, and 5×10^{-5} for plutonium concentrations of 10^{-7} M, 10^{-6} M, and 10^{-5} M, respectively. Here, an order of magnitude increase in plutonium concentration from 10^{-6} M to 10^{-5} M resulted in a 2000-fold decrease in the fractional survival of *C. heintzii*.

In the plutonium-239 experiments, the source of ionizing radiation was the alpha particle decay of the actinide. The average energy of this alpha particle is 5.15 MeV. Unlike gamma radiation, alpha particles deposit their energy in aqueous solution in dense tracks that have a range of ~ 40 μm and a diameter of ~ 1 μm (Draganic and Draganic, 1971). This leads to a nonhomogeneous distribution of radiolytic products and a relative increase in the yield of molecular products (e.g., H_2O_2 and H_2) at the expense of the more transient free radical species (OH, H, and e_{aq}). Cell death is caused by the reaction of these transients with the suspended cells, the reaction of scattered radiation that is deposited in the cellular material (spurs within the cell), and the interactions between the transients generated in the alpha tracks within the cell. When the alpha particles interact directly with cellular material, the observed rate of cell death is expected to be higher than projected, based on radiation tolerance, because the in-homogeneity of the energy deposition leads to a relatively high amount of non-repairable damage to cellular DNA. In other words, to properly account for damage done by alpha particles, we have to distinguish between the radiolytic energy deposited directly into the cells – which causes much more damage to cellular material – and energy that is deposited outside the cellular materials.

We cannot account, solely on the basis of dose-to-solution, for the enhanced loss of viability observed when ^{239}Pu is the source of ionizing radiation. In the *C. heintzii* experiments, the time- and space-averaged dose rate for 10^{-5} M plutonium was 0.016 Gy/h (1.6 rad/h). This corresponds to a total absorbed dose of 2.7 Gy for a seven day experiment which is only a small fraction of the absorbed gamma dose needed to cause significant loss of viability (approximately 1.5% of that needed for 1% survivability). This suggests that either the mechanism by which ionizing radiation causes cell death is not the same, and/or the radiation dose received by the suspended cells is not accurately predicted by assuming a homogeneous distribution of the source of alpha particles.

Radiotoxicity of plutonium-242 toward C. heintzii

Radiotoxicity experiments were performed with $1.0 \pm 0.1 \times 10^{-5}$ M ^{242}Pu (see Tables 2 and 3). This plutonium concentration is comparable to the total plutonium concentration used in the highest-concentration ^{239}Pu experiments. Its activity is comparable to the $\sim 10^{-6}$ M ^{239}Pu experiments, since the ^{242}Pu used was 7.8 times less radioactive than the ^{239}Pu used. This activity ratio is a factor of two less than the theoretical value of 15.6 because of the presence of ^{238}Pu impurity in the ^{242}Pu stock solution.

The fractional survival of *C. heintzii* in the ^{242}Pu-NTA experiments, along with those obtained for the 10^{-5} M and 10^{-6} M ^{239}Pu experiments are shown in Figure 4. Initially, at times less than 50 h, no significant differences in the loss of cell viability were noted. When the survival data for the same-concentration Pu experiments are compared, a significant increase in the loss of cell viability for the higher activity ^{239}Pu

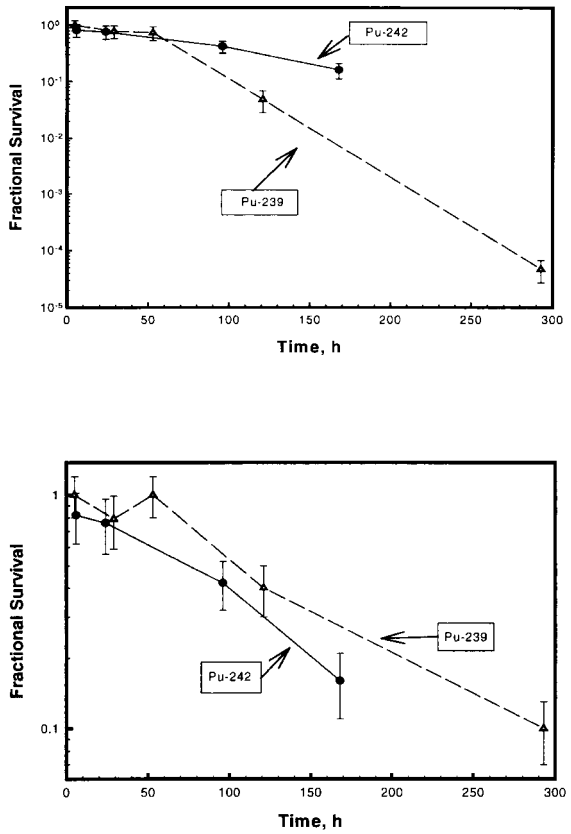

Figure 4. Comparison of fractional survival data for *C. heintzii* in the Pu(IV)-NTA-*C. heintzii* system. Top: Fractional survival for 10^{-5} M ^{242}Pu (●) and 10^{-5} M ^{239}Pu (▲), showing greatly increased loss of viability for the higher activity ^{239}Pu. Bottom: Fractional survival for 10^{-5} M ^{242}Pu (●) and 10^{-6} M ^{239}Pu (▲), showing the same effect.

experiments is noted. After four days, the fractional survival of *C. heintzii* was 3.5 times greater for ^{242}Pu vs. ^{239}Pu. This difference increased to a factor of ∼27 after seven days.

A comparison of the data for 10^{-6} M ^{239}Pu and 10^{-5} M ^{242}Pu, which are approximately equal in activity, shows that the fractional survival of *C. heintzii* was the same, within experimental error (Figure 4). This persisted over the two-week timeframe the experiments were monitored.

The results obtained in the variable-isotope studies support the hypothesis that the primary cause of the observed plutonium toxicity was ionizing radiation, not chemical toxicity. This is supported by the significant differences observed in the radiotoxity of the same-concentration (∼10^{-5} M) ^{239}Pu and ^{242}Pu experiments, and by the similar toxicity effects noted for experiments with the same alpha activity. The predominance of radiolytic effects for ^{239}Pu is consistent with observations made by others (Wildung et al., 1987; Wildung and Garland, 1980; Wildung and Garland, 1982).

Relationship between bioassociation and radiotoxicity

The dependence of radiotoxicity on the rate of linear energy transfer (LET) is well established (Ewing, 1987). Higher LET ionizing radiation (e.g., alpha particles and recoil nuclei) deposit their energy in dense tracks that result in high concentrations of broken bonds. This, in turn, leads to a higher probability of new bond formation, hence un-repairable cell damage to the bacteria. This effect is clearcut when cellular mass is being irradiated. The situation is significantly different, however, when the cells are suspended in aqueous media (e.g., our experiments). Here, much of the ionizing radiation is deposited in the media rather than directly in the cellular mass. In this situation, there are two radiolytic contributions to cell death: (1) reaction of radiolytic transients generated in bulk solution with cellular material, and (2) reaction of radiolytic transients generated within the cell due to direct interactions between the high LET alpha particle and cellular mass.

An important observation we have made in our Pu(IV)-NTA-*C. heintzii* study is that dose-to-solution (see Table 2), when plutonium is the source of ionizing radiation, does not account for the observed loss of viability. This suggests that the bioassociation of plutonium with *C. heintzii* is a necessary and important step in accounting for the observed radiotoxicity. This greatly enhances the probability of direct interactions between the alpha particle and the bacterium, which we believe to be the primary cause of cell death.

The reactions of the radiolytic products generated in the aqueous medium with cellular mass do not account for the observed loss of cell viability in our experiments. Past research (Ewing, 1987) has identified the oxidizing primary radiolytic products ($OH^{\cdot}$, H_2O_2, and $HO_2^{\cdot}$), as the key transients responsible for the observed loss of cell viability. There are two sets of data that help establish the radiolytic contribution to the loss of cell viability due to interactions with the aqueous medium: These are: (1) the results of our gamma irradiation studies and (2) the observed effects when the plutonium existed as a dissolved Pu(IV)-NTA complex.

In the highest-activity system we investigated (9.9×10^{-6} M ^{239}Pu), there were $6.0 \pm 1.5 \times 10^7$ cells/mL, 5.96×10^{15} plutonium atoms/mL, and 1.93×10^7 alpha particles generated/mL/h. This corresponds to 9.9×10^7 plutonium atoms/bacterium and 0.32 alpha particle disintegrations/bacterium/h. There are two reasons why contributions to loss of cell viability would not be expected from alpha decays originating from plutonium-NTA complexes in the bulk solution. First, total absorbed dose, adjusted for differences in high/low LET yields, only accounts for ~1% of the observed loss of cell viability. Second, the likelihood of direct interaction between the 1 μm diameter by 40 μm alpha particle track with *C. heintzii* is <1% because a single *C. heintzii* cell occupies only 0.5 μm^3 of each 1×10^4 μm^3 of solution (on average).

The potential contribution to loss of cell viability from interactions with bulk solutions can also be estimated from the gamma irradiation studies. Net yields of oxidants (Draganic and Draganic, 1971) for low LET radiation (0.23 keV/μm for 1 MeV gamma radiation) are G_{OH} = 2.9 molec/100 eV, $G_{H_2O_2}$ = 0.86 molec/100 eV, and G_{HO_2} = 0 molec/100 eV. This compares to yields of G_{OH} = 0.3 molec/100 eV, $G_{H_2O_2}$ = 1.3 molec/100 eV, and G_{HO_2} = 0.35 molec/100 eV for high LET radiation (~130 keV/μm for 5 MeV alpha particles). Net yield of oxidants are in fact lower for high LET radiation, with the OH yield almost a factor of ten lower and the H_2O_2 yield within a factor of two.

Lastly, the increased rate of cell death appears to be correlated with the bioassociation of plutonium (see Figure 5). The bioassociation of plutonium, in the high-concentration plutonium experiments, depends on the availability of NTA to form a Pu(IV)-NTA complex. Plutonium atoms absorbed onto the cell wall have a 50% likelihood of interacting directly with the cell. Those within the cell have a 100% chance of interaction with the cell.

The hypothesis that direct interaction of alpha particle tracks with cellular material lead to certain loss of viability was evaluated by calculating the rate of cell death under a variety of conditions (see Figure 6). When no bioassociation is present, dose-to-solution (based on radiation tolerance) and random alpha particle interactions estimate cell death (where alpha particle tracks at random interact with cells). In the presence of bioassociation, the rate of cell death is estimated under two conditions: (1) bioassociation on the cell wall (50% interaction with the cell) and (2) bio-uptake (100% interaction with the cell). The calculated rate of cell death is ~2×10^5 cell deaths/h/mL for 1% association at 0.01 mM Pu-239 concentrations. The data reflected in Figure 6, within the uncertainties of the cfu counts made, support our hypothesis that direct interactions between the alpha particle tracks and the cellular material account for the observed loss of viability.

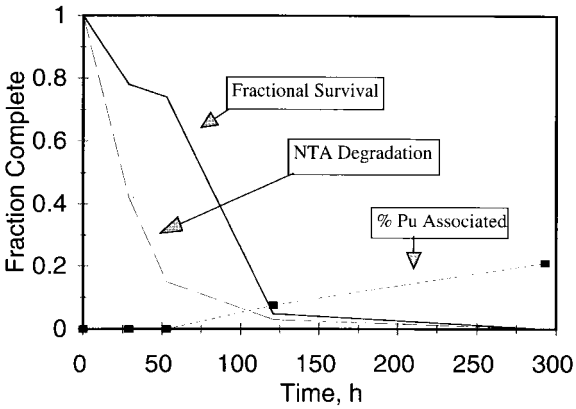

Figure 5. Relationship between NTA degradation (– – –), association of plutonium (- - -), and fractional survival (—) in the Pu(IV)-NTA, *C. heintzii* system at [Pu] = 10^{-5} M. Increased plutonium toxicity is correlated with the bioassociation of plutonium.

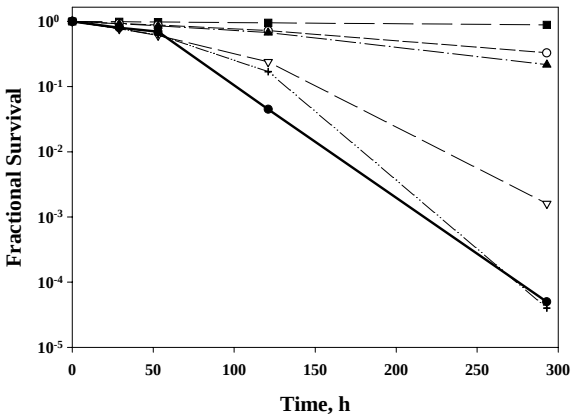

Figure 6. Contribution of radiolytic effects to loss of *C. heintzii* viability from dose-to-solution (■), random α-particle interaction (○), and sum of these two contributions (▲), including effects of surface-adsorbed Pu (▽) and assuming bio-uptake (+). The measured loss of viability is given by the solid curve (●). All data correspond to 10^{-5} M concentrations of Pu-239.

Conclusions

The interaction of ionizing radiation, whether it be gamma rays or alpha particles, with bacteria resulted

in the loss of microbial viability. For *C. heintzii*, the gamma radiation tolerance was 165 ± 30 Gy, which is typical of prokaryotic bacteria in oxygenated systems. The toxicity of plutonium was primarily due to radiolytic effects associated with the interactions of the alpha particle emitted. The observed toxicity, however, was not due to bulk solution effects. The radiolytic effects were linked to the bioassociation of plutonium with *C. heintzii*. It is proposed that this relationship exists because direct interactions between the alpha particle track and the bacterium lead to a high probability of cell death. The estimated contribution of direct alpha particle interactions to cell death account for the experimentally observed rate of cell death. It is therefore important to account for the distribution of alpha particle emitters (e.g., Pu-239 in our study) to establish its radiotoxicity when implementing a bioprocessing or bioremediation technology.

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