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Effects of Plutonium on Soil Microorganisms

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As a first phase in an investigation of the role of the soil microflora in Pu complex formation and solubilization in soil, the effects of Pu concentration, form, and specific activity on microbial types, colony-forming units, and CO_2 evolution rate were determined in soils amended with C and N sources to optimize microbial activity. The effects of Pu differed with organism type and incubation time. After 30 days of incubation, aerobic sporeforming and anaerobic bacteria were significantly affected by soil Pu levels as low as 1 μg/g when Pu was added as the hydrolyzable $^{239}Pu(NO_3)_4$ (solubility, <0.1% in soil). Other classes of organisms, except the fungi, were significantly affected at soil Pu levels of 10 μg/g. Fungi were affected only at soil Pu levels of 180 μg/g. Soil CO_2 evolution rate and total accumulated CO_2 were affected by Pu only at the 180 μg/g level. Because of the possible role of resistant organisms in complex formation, the mechanisms of effects of Pu on the soil fungi were further evaluated. The effect of Pu on soil fungal colony-forming units was a function of Pu solubility in soil and Pu specific activity. When Pu was added in a soluble, complexed form [$^{238}Pu_2$(diethylenetriaminepentaacetate)$_3$], effects occurred at Pu levels of 1 μg/g and persisted for at least 95 days. Toxicity was due primarily to radiation effects rather than to chemical effects, suggesting that, at least in the case of the fungi, formation of Pu complexes would result primarily from ligands associated with normal (in contrast to chemically-induced) biochemical pathways.

Plutonium is present in surface soils in low concentrations (0.07 to 0.4 pg/g) as a result of worldwide fallout from nuclear testing (5). There is also a possibility that Pu may enter soils as a result of the operation of a nuclear fuel cycle, such as in airborne particulates or liquid effluents from fuel fabrication, reprocessing, or waste storage facilities. These factors, coupled with the long half-life of Pu, have necessitated estimation of its dose-to-man over thousands of years. A major uncertainty in this evaluation is the effect of soil processes on Pu solubility in soil and plant availability over a long period of time (12).

Plutonium is initially largely insoluble in soils and sediments (3, 4), probably because of the relative stability of the Pu(IV) valence state and its tendency to form insoluble hydrolysis products in aqueous solutions of low acidity. However, over a long period of time, soil microorganisms might directly or indirectly play a role in the solubilization of hydrolyzed Pu in soils. This may occur through effects on the soil physicochemical environment, e.g., pH and Eh, which lead to increased chemical solubility or to the formation of Pu(V) and Pu(VI), which, from a thermodynamic standpoint, are less subject to hydrolysis, and through effects on the levels of soluble inorganic ligands (e.g., HCO_3^-, CO_2^{2-}) or organic ligands capable of stabilizing Pu(IV) against hydrolysis and maintaining increased levels of Pu in the soil solution. The latter effect has been demonstrated in vitro; that is, soil fungal isolates have been shown to produce metabolites that form complexes with Pu and Ni, reducing their sorption upon elution through soil (10, 11). Formation of complexes with organic ligands may occur through interactions of Pu with products of the normal metabolism of soil microorganisms or as a result of a specific protective action by the organism to convert Pu to a form which is either less capable of entering the cell or is not chemically toxic to the cell. The type of complex formed may thus be dependent upon the mechanism of toxicity of the element.

As a first phase in an investigation of the role of soil microorganisms in the formation of Pu complexes in soil, experiments were undertaken to determine the effects of Pu concentration on the growth of bacteria, fungi, and actinomycetes in soils and on overall microbial activity as measured by soil CO_2 evolution. To examine the effects of Pu solubility on the soil microflora and to separate possible chemical effects from radiation effects, important factors in distinguishing possible mechanisms of complex formation, microbial colony-forming units (CFU), and CO_2 evolution were measured in soils to which Pu

was added in complexed and uncomplexed forms differing in soil solubility and in two isotopes differing in specific activity.

MATERIALS AND METHODS

Soil preparation and amendments. A Ritzville silt loam surface (0 to 15 cm) soil was used for all studies. After the soil was sampled in sufficient quantity, it was screened (5 mm), air dried (approximately 8% moisture), thoroughly mixed, and stored at 25°C. Subsamples were removed by quartering for subsequent microbial studies. The soil was noncalcareous, had a pH of 6.8, and contained 0.7% organic C. All results are reported on the basis of oven-dried (110°C) soil.

Standard solutions of $^{239}Pu(NO_3)_4$ and $^{238}Pu(NO_3)_4$ prepared in 2.0 M HNO_3 and [$^{238}Pu_2$(diethylenetriaminepentaacetate)$_3$] {[$^{238}Pu_2$(DTPA)$_3$]} were employed for soil amendments. The total α activity of the ^{239}Pu standard solution was due primarily to ^{239}Pu but, in addition, the solution contained other isotopes, including ^{240}Pu (21.2%), ^{238}Pu (5.8%), and ^{241}Am (4.0%), which contributed to α activity. Because this distribution varied in stock solutions, depending upon reactor operating conditions and storage time, the mass of Pu at a given α activity level varied somewhat between treatments. However, each new stock solution was assayed, and variation did not exceed 20%. The ^{238}Pu solution contained 99.91% ^{238}Pu on an α activity basis. The Pu isotopes in all solutions constituted 99.9% of the radioisotopes present in solution on a weight basis.

A subsample which constituted <5% of the air-dried soil to be employed at each treatment level was removed and mixed with sufficient $CaCO_3$ (<0.4 g) to neutralize the HNO_3 to be added in the Pu standard solution and with the standard solution. The amended soil was dried (4 h, 60°C) and thoroughly mixed (4 h) in a V-blender with the original air-dried soil to be used at each treatment level. Final Pu levels in soil were 0.05, 0.5, and 10 μCi of ^{239}Pu per g of soil (corresponding to ca. 1, 10, and 180 μg of Pu per g of soil, respectively) and 10 μCi of ^{238}Pu per g (corresponding to 0.6 μg of Pu per g of soil). After dry mixing, the soil was mixed with 1% starch and 0.5% NH_4NO_3 to ensure sufficient microbial activity for assessment of Pu effects and sufficient H_2O to bring the soil to 22% H_2O. Controls consisted of similar treatments without Pu and with and without DTPA added in quantities equivalent to $^{238}Pu_2$ (DTPA)$_3$ treatments. Duplicate subsamples of soil (200 g) at each treatment level were subsequently placed in incubation vessels for microbial studies.

Microbial studies. Two methods were employed to determine the effect of Pu on microorganisms in the soil. These included estimation of viable organisms by plate counts and of metabolic activity as indicated by CO_2 evolution rate. Measurements were conducted concurrently with the same incubation system. Soil samples (200 g [dry weight]) were incubated in duplicate in glass incubation cells (500 cm^3) in the dark at 28°C for up to 95 days. The cells were stoppered and continuously flushed with moistened (H_2O bubbler) CO_2-free (Ascarite; Arthur H. Thomas Co.) air. The soil samples were subsampled periodically by insertion and withdrawal of a glass tube through a sampling port in the top of the incubation cell. Each subsample of soil (1 g [dry weight]) was placed in 1 liter of sterile distilled H_2O and shaken. Appropriate soil dilutions were made from this stock inoculum. Aliquots (1 ml) of the diluted stock solutions were subsequently cultured in triplicate on selective media, and CFU were determined by plate counts after incubation for 6 days at 28°C. Bacteria were cultured on soil extract agar (7). Aerobic and microaerophillic bacteria were differentiated from anaerobic and facultative anaerobic bacteria by aerobic and anaerobic incubation. Sporeforming bacteria were distinguished from non-sporeforming bacteria by heat inactivation. Fungi were cultured on oxgall agar containing 30 μg of streptomycin per g (6). Actinomycetes were cultured on glycerol-asparagenate (1).

The CO_2 in the soil effluent gases, an index of respiration rate, was absorbed in NaOH contained in Pettenkofer tubes and analyzed (8) by titration of the unneutralized NaOH after precipitation of CO_3^{2-} as the Ba salt. A continuously recording automatic titrimeter was used. To improve the sensitivity of the method, we adjusted the concentrations of NaOH in accordance with the expected CO_2 evolution rate (9). Concentrations of HCl used for the titration of unneutralized base were also adjusted accordingly.

Plutonium solubility. The solubility of Pu was measured at periodic intervals during incubation to assess the effect of this parameter on the microbial population. A soil suspension was prepared by thoroughly mixing soil (1 g) that was subsampled from the incubation cell as described above in H_2O (100 ml) for 10 min. After equilibration for 48 h, a portion (~50 ml) was successively filtered through 5-, 0.45-, and 0.01-μm membrane filters (Millipore Corp.). The Pu in the filtrates from the <0.01-μm filters was operationally defined as water soluble, although it was recognized that Pu in the soil amended with $Pu(NO_3)_4$ was readily hydrolyzable and may have been present, in part, as colloids <0.01 μm in diameter (4).

RESULTS

The effect of Pu concentration on the growth of all classes of organisms examined is summarized in Fig. 1. The effect of Pu relative to controls differed with organism type and incubation time. With the exception of the aerobic bacteria and actinomycetes, the effects were most pronounced at the end of the 30-day incubation period. In the case of the aerobic bacteria, the end of the exponential growth phase for controls and treated soil occurred at approximately 11 days, and the CFU for the controls were decreasing at 30 days. The actinomycetes appeared to undergo pronounced successional changes reflected in two exponential growth phases, beginning at 2 and 12 days, in controls and treated soils over the 30-day period. The second exponential growth phase was complete at 30 days. These phenomena resulted in significant effects on growth early in, but not at the end of, the 30-day incubation period. Other classes of organisms generally achieved maximum growth over a 6- to 15-day incubation period, and controls remained relatively constant there-

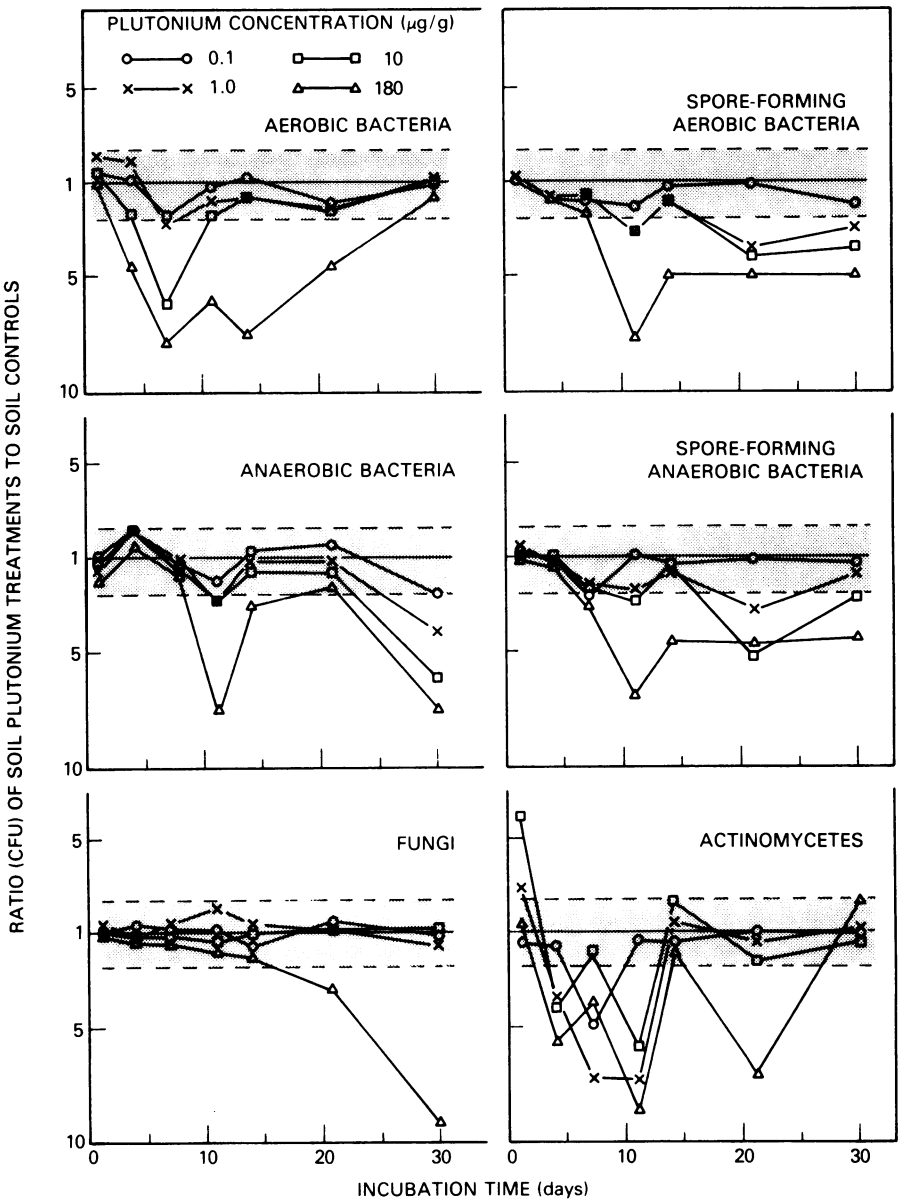

FIG. 1. Influence of ^{239}Pu concentration (added as $^{239}Pu(NO_3)_4$) on the growth of microflora in soil. Values outside the shaded areas are significantly different from control values ($P < 0.05$).

after. After 30 days, the sporeforming aerobic bacteria and anaerobic bacteria were sensitive to Pu at levels as low as 1 μg/g. Sporeforming anaerobic bacteria were sensitive (growth or sporulation or both) to Pu at levels of 10 μg/g, whereas 180 μg of Pu per g was required to significantly reduce fungal CFU relative to controls, and effects at this level were not evident until after 20 days of incubation.

Accumulative CO_2 evolved during the incubation period generally reflected the pattern of microbial growth, with maximum CO_2 evolution rates occurring during days 12 to 14 of incubation. The rates of CO_2 evolution and CO_2 accumulation over this incubation period were significantly reduced relative to controls (total CO_2 evolved, 1,214 mg) only by 180 μg of Pu [as $Pu(NO_3)_4$] per g (total CO_2 evolved, 973 mg), although the numbers of all classes of organisms, except the fungi, were depressed below this level (Fig. 1).

To distinguish between the effects of Pu sol-

ubility and chemical versus radiation effects on the soil microflora, we examined the growth of fungi, which exhibited the greatest resistance to Pu in soil, in a separate experiment in which soils were amended with complexed and uncomplexed forms of Pu differing in solubility and with Pu isotopes differing in specific activity.

The DTPA complexes of ^{239}Pu and ^{238}Pu were largely water soluble (<0.01 μm) at all concentrations over an extended incubation period in soil, with Pu solubility decreasing approximately 20% after 95 days, perhaps as a result of the degradation of the organic moiety and subsequent Pu hydrolysis. In contrast, after approximately 2 h of incubation, hydrolysis of Pu added to soil as $Pu(NO_3)_4$ resulted in Pu water solubilities <0.1% of that initially applied. These differences in form and solubility influenced Pu toxicity for the fungi relative to controls (Table 1).

The controls, containing starch, N, and DTPA only, exhibited increases in fungal numbers with time up to 25 days as growth and reproduction occurred (Table 1). By 95 days, fungal numbers had decreased approximately 50% but were well above initial levels (at day 0). Of interest was the observation that the controls receiving DTPA, as well as starch and N, had slightly higher CFU, suggesting that DTPA had a stimulatory effect on growth. Because DTPA did not contribute a significant increase in organic C and because Pu solubility studies indicated that only 20% of the DTPA was degraded over this period, the stimulatory effect may, therefore, have resulted from DTPA solubilization of nutrient elements in soil.

Increasing the levels of ^{239}Pu-DTPA resulted in significantly lower fungal CFU, relative to DTPA controls, at all concentration levels tested after 11 days of incubation (Table 1). As observed in the previous experiment (Fig. 1), the Pu added to soil as $Pu(NO_3)_4$ at the 180 μg/g level reduced fungal CFU after 25 days of incubation relative to controls containing starch and N only. However, $Pu(NO_3)_4$-treated soil (180 μg of ^{239}Pu per g and 0.6 μg of ^{238}Pu per g) were at least one order of magnitude less toxic after 11 days, relative to controls, than ^{239}Pu-DTPA-treated soil at the same mass levels, indicating the importance of Pu solubility for toxicity.

Differences in specific activity between ^{239}Pu and ^{238}Pu allowed differentiation between chemical effects on microbial CFU. In soils receiving ^{238}Pu-DTPA (0.6 μg/g) and ^{239}Pu-DTPA (180 μg/g), fungal CFU were markedly reduced, relative to controls, after 11 days of incubation, but the effects of the treatments were approximately equivalent (Table 1). These treatments differed in Pu concentration by a factor of 300 but had equivalent radioactivity levels (10 μCi/g), indicating that toxicity was due to radiation effects rather than to chemical effects. The analogous comparison of $^{239}Pu(NO_3)_4$- and $^{238}Pu(NO_3)_4$-treated soil at the 10-μCi/g level indicates the same phenomenon, but toxicity at both mass levels was not as pronounced as in the DTPA treated soils, reflecting the importance of Pu solubility, as well as radiation level, on toxicity.

DISCUSSION

The possible mechanisms of Pu solubilization in soil over a long period of time may be related to the nature and extent of Pu toxicity for soil microbial populations. Organisms resistant to

TABLE 1. Effect of concentration, form, and specific activity of Pu on fungal CFU in soil

Treatment[a] (μg/g)	Fungal CFU (× 10^5)[b] at incubation time of:				
	0 days	4 days	11 days	25 days	95 days
Controls					
Starch, nitrogen only	0.37 ± 0.09	0.57 ± 0.09	0.94 ± 0.08	3.05 ± 0.45	1.18 ± 0.31
Starch, nitrogen, DTPA (180)	0.37 ± 0.08	1.26 ± 0.37	2.54 ± 0.31	4.74 ± 0.93	1.78 ± 0.23
^{239}Pu					
Pu-DTPA (1)	0.33 ± 0.04	0.63 ± 0.07	1.01 ± 0.12	2.41 ± 0.85	1.06 ± 0.16
Pu-DTPA (10)	0.36 ± 0.05	0.57 ± 0.12	0.87 ± 0.21	1.22 ± 0.12	0.08 ± 0.013
Pu-DTPA (180)	0.32 ± 0.05	0.53 ± 0.07	0.07 ± 0.014	0.07 ± 0.013	0.003 ± 0.0015
Pu $(NO_3)_4$ (180)	0.37 ± 0.03	0.76 ± 0.11	0.76 ± 0.19	1.29 ± 0.74	0.25 ± 0.03
^{238}Pu[c]					
Pu $(NO_3)_4$ (0.6)	0.36 ± 0.05	0.57 ± 0.18	0.45 ± 0.16	0.33 ± 0.07	0.02 ± 0.003
Pu-DTPA (0.6)	0.38 ± 0.04	0.21 ± 0.12	0.08 ± 0.012	0.04 ± 0.006	0.004 ± 0.002

[a] Pu-treated soils received starch and N at levels equivalent to those used for controls.
[b] Mean of two replicates (three measurements per replicate) ± standard deviation.
[c] The specific activity of ^{238}Pu is a factor 300 greater than that of ^{239}Pu.

elevated levels of Pu in soil may play a special role in complex formation and solubilization processes. These organisms may have a greater possibility for indirectly influencing Pu solubility through formation of complexes with normal exocellular metabolites, as they may have a competitive advantage and be present in larger numbers where localized Pu concentrations occur, such as at the surface of soil colloids. Furthermore, it is important to understand the mechanism of resistance to a radioisotope, because chemical toxicity may result in the development of special pathways to limit cell transport or in effects at the cytoplasmic or exocytoplasmic levels. These may involve alteration of the form and, perhaps, of the solubility of Pu.

In the present studies, the soil CO_2 evolution rate, a measure of overall microbial activity, was significantly affected only at ^{239}Pu levels of 180 μg/g in soil when ^{239}Pu was added in the hydrolyzable form $[Pu(NO_3)_4]$. This is in contrast to results of studies with other heavy metals, such as Ag, Hg, and Cr (H. Drucker, T. R. Garland, and R. E. Wildung, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, N2, p. 163; H. Drucker, R. E. Wildung, T. R. Garland, and M. P. Fujihara, Abstr. Annu. Meet. Am. Soc. Agron. 1973, p. 90), in which soil CO_2 evolution rate was a sensitive measure of the effects of metals at levels as low as 1 μg/g. Microbial CFU was a far more sensitive measure of effects, particularly considering the very low solubility of Pu in soil after hydrolysis. Microbial CFU differed with organism type and incubation time. After 30 days of incubation, all classes of organisms, except the fungi, were affected at concentrations of 10 μg of Pu per g of soil, and concentrations as low as 1 μg of Pu per g of soil affected the aerobic sporeforming and anaerobic bacteria.

Because of their resistance and, therefore, their possible importance in complex formation, the fungi were examined in subsequent investigations to evaluate the mechanisms of effects. These investigations indicated that effects were a function of Pu solubility in soil and that toxicity was more pronounced when soluble, complexed forms were added to soil. Under these circumstances, effects on the fungi were markedly increased, were accentuated with incubation time, and persisted for at least 95 days.

At the soil Pu levels employed, which exceeded current fallout levels by several orders of magnitude, toxicity for the fungi appeared to be due primarily to radiation effects rather than to chemical effects. In contrast to chemical resistance, radiation resistance is associated with an ability to repair radiation damage to key macromolecules without development of new biochemical pathways which might alter Pu solubility in soil. This suggests that, at least in the case of the fungi, any alteration of Pu solubility or form in soil would be by indirect mechanisms, that is, formation of complexes with ligands arising from normal metabolism. However, this would not exclude the possibility of responses by organisms not contributing significantly to total numbers, i.e., not detected by the assessment methods employed, or the possibility of microbial selection with time. These possibilities are currently being examined in pure culture systems with defined media and with organisms isolated from soil by Pu enrichment techniques.

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