

Biological Reduction and Removal of Np(V) by Two Microorganisms

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The majority of the radionuclides generated by the nuclear fuel cycle can be removed during established remediation processes. However among the long-lived, α -emitting actinides neptunium(V) is recalcitrant to removal from solution by physicochemical or biotechnological methods. The latter include a biocrystallization process, based on the enzymatic liberation of phosphate as a precipitating ligand by a *Citrobacter* sp., which was previously shown to precipitate tetravalent actinides such as Th(IV) and Pu(IV) as their corresponding phosphates. Np(V) was reduced to a lower valence (probably Np(IV)) by ascorbic acid or biologically, using the reductive capability of *Shewanella putrefaciens*, but reduction alone did not desolubilize Np. However Np(V) was removed by the two organisms, *S. putrefaciens* and *Citrobacter* sp. in concert; bioreduction to Np(IV) by *S. putrefaciens*, together with phosphate liberation by the *Citrobacter* sp., permitted bioprecipitative removal of ^{237}Np as well as its daughter $^{233}\text{protactinium}$. Tests were made possible by a novel technique permitting actinide separation by paper chromatography followed by quantification of the radioactive species using a phosphorImager. This study has implications for the development of methods to remove Np(V) from solution, by the simple combination of two biotechnological methods, which can succeed where chemical treatments are ineffective.

Introduction

In addition to the problem of extant radioactively contaminated sites, the stringent legislative requirements and current concerns over the potential fate of "nuclear" wastes prompts evaluation and implementation of new technologies for the removal of contaminants from wastes and from the biosphere. The long-lived α -emitting actinides are problematic, attributable to their very long half-lives and high radiotoxicity; these factors make their effective removal at the source both urgent and imperative.

Among the transuranic nuclear fuel cycle elements neptunium is problematic. It occurs in low-active wastes (1, 2) and, after uranium, can be the predominant radionuclide (1, 2). It arises via decay of $^{241}\text{plutonium}$ (half-life: 14.9 years) and $^{241}\text{americium}$ (half-life: 433 years) to stable $^{237}\text{neptunium}$ (half-life: 2.1×10^6 years). Eventually $^{237}\text{neptunium}$ will predominate in historical releases (2). While plutonium(IV) forms insoluble complexes in soils and sediments, limiting environmental mobility and biological availability, neptunium(V) complexes ligands poorly (3–5) and, as the α -emitting

NpO_2^+ cation, is highly mobile, biologically available, and very difficult to remove from solution by established methods. Prompted by early studies using uranium (6) and available biotechnology for radionuclide remediation (2) but previously constrained by the chemistry of neptunium(V) (4, 5), we describe a novel coupling of biological reduction of neptunium using *Shewanella putrefaciens* (previously documented to reduce uranium (VI) (7, 8) and technetium (VII) (9)) to a phosphate-based biocrystallization method for uranium (10, 11), plutonium, and americium (11) which was ineffective alone against neptunium (V) (11). These studies were prompted by the observation that *S. putrefaciens* can use uranium as a terminal electron acceptor anaerobically, with concomitant reduction of U(VI) to U(IV) (12) and can also reduce Fe(III) to Fe(II) anaerobically (13). The redox potentials for the appropriate couples are as follows: $\text{UO}_2^{2+}/\text{U}^{4+} = 0.32$ V; $\text{NpO}_2^+/\text{Np}^{4+} = 0.739$ V; $\text{Fe}^{3+}/\text{Fe}^{2+} = 0.771$ V (14); it is likely that Np(V) would be reduced similarly. Chemically, both ferrous ammonium sulfate and ascorbic acid can reduce Np(V) to the tetravalent state (14). It is likely that Np(IV) could be precipitated as the phosphate; the phosphates of the tetravalent actinides are well documented in the literature (4, 15), and previous studies have shown that thorium(IV) phosphate can be removed by a phosphate-liberating *Citrobacter* sp. (16). Actinide elements in the same oxidation state have very similar properties (17), and there are also many similarities in the chemical properties of the trivalent actinide and lanthanide elements (18).

In a model system with La(III) and Th(IV) as "surrogates" for ^{241}Am (III) and ^{239}Pu (IV), and in tests using the corresponding active nuclide species, metals were removed from solution as cell-bound phosphate biomineral, using precipitant HPO_4^{2-} ligand released via the activity of a cell-bound phosphatase of *Citrobacter* sp. (11). Immobilized *Citrobacter* cells in a column removed >90% of the input metal, to a load of 9 g of U/g of biomass dry weight (19), as HUO_2PO_4 (10). In contrast to this, and to the corresponding removal of La and Th phosphates (16, 20), Np(V) phosphate is fairly soluble (4, 5), and Np was not removed by this method alone (11). Removal of Np by intercalation into pre-existing polycrystalline uranyl or lanthanum phosphate is possible (21, 22), but such intercalation is restricted to Np(VI) (23), which arises in solution via disproportionation of Np(V), and the rate of this would be limited by the disproportionation rate of Np(V) in solution and the equilibrium position; in neutral aqueous solution Np(V) predominates (24–26).

Since the removal of Np(V) via both the biocrystallization and intercalation routes is subject to chemical constraints, we reasoned that if a biological system could reduce Np(V) to Np(IV), the latter could be removed via bioprecipitation, as for Pu(IV) and Th(IV) (11, 27). Illustration of this formed the purpose of the study.

Materials and Methods

Microorganisms and Culture Conditions. *Citrobacter* sp. (10, 11, 16, 27) was used by permission of Isis Innovation, Oxford, U.K. The organism was grown with vigorous aeration (30 °C) in glycerol–glycerol 2-phosphate-based minimal medium, with biomass harvest and storage as previously described (16, 27, 28). Cells were suspended for use at a concentration of 0.47 mg dry weight mL^{-1} in 40 mM MOPS–NaOH buffer supplemented with 5 mM glycerol 2-phosphate (organic phosphate donor) and 100 mM ammonium acetate (pH 7) (to enhance the efficiency of actinide phosphate bioprecipitation (16, 28)).

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Shewanella putrefaciens (ATCC 8071) was obtained from Dr. D. R. Lovley (University of Massachusetts) and was grown in the defined medium of Lovley and Phillips (29) under an anaerobic atmosphere consisting of N₂ and CO₂ (80:20). Sodium lactate (100 mM) and ferric citrate (50 mM) were supplied as electron donor and acceptor, respectively. All manipulations of *S. putrefaciens* were done under N₂/CO₂. Prior to use, cells were collected from early stationary phase cultures by centrifugation and washed three times in 40 mM MOPS–NaOH as described by Lloyd and Macaskie (9). For use in bioreduction tests cells were resuspended at a concentration of 0.31 mg mL⁻¹. In some tests, cells were washed three times and resuspended in carbonate buffer (2.5 g NaHCO₃, 1.5 g NaH₂PO₄, 0.1 g KCl per L distilled water) and deaerated with 80%/20% N₂/CO₂. Where both organisms were used together (biomass content as above) the carrier was 40 mM MOPS–NaOH with glycerol 2-phosphate and ammonium acetate as above (pH 7). All cell suspensions (2 mL final volume) were contained in 12 mL serum bottles (Adelphi Tubes Ltd., Hayward's Heath, Sussex, U.K.) at 30 °C.

Chemicals and Radiochemicals. ²³⁷Np (containing daughter β-emitter ²³³Pa) used for most experiments was from AEA Fuel Services, Oxfordshire, U.K. ²³⁹Np (γ-emitter) was a gift from BNFL (Salwick, Preston, U.K.) and was added to a carrier solution of ²³⁷Np (²³³Pa free; purified by ion-chromatography by BNFL). The use of ²³⁹Np required specialist facilities at BNFL for its preparation and detection and it was difficult to use routinely because of its short half-life (2.35 days). The ^{239/237}Np mix was used within 3 days of preparation, before ²³³Pa was detectable. La, Th, and U nitrates were from BDH (Poole, Dorset, U.K.), and other chemicals were from Sigma.

Reduction of Np(V) by Washed Cell Suspensions and Ascorbic Acid. ²³⁷Np (α-emitter containing daughter β-emitter ²³³Pa; concentration of ²³³Pa at use date was not determined) was added aseptically from an anaerobic stock to a final concentration of 100 μM. ²³⁹Np (γ-emitter), when used, was supplied to the cells at a concentration of 2 pM, in a carrier solution of 2 μM ²³⁷Np, from which the ²³³Pa daughter element had been removed (above). Hydrogen was supplied in the headspace of the bottles when required as an electron donor for Np reduction. Np(V) was reduced chemically as required by the addition of ascorbic acid to a final concentration of 1% (wt/vol) in cell-free control experiments.

For bioreduction tests cells were incubated anaerobically (24 h) in the presence of either 100 μM ²³⁷Np or 2 μM ²³⁷Np/2pM ²³⁹Np as above. Cells and supernatant were separated by centrifugation, and the residual species in the supernatant (3 × 10 μL spots, dried under N₂ between additions: total load 30 μL) were separated chromatographically and quantified as described below.

Chromatographic Separation of Tri, Tetra- and Pentavalent Actinides. Different oxidation states of the actinides Np and Pa were separated chromatographically using Whatman 3MM paper (prewashed with 2 M HCl and several changes of distilled water). The mobile phase was trimethylamine/acetone/formic acid 2:6:2 (v/v/v). The method was calibrated using “surrogate” elements La(III), Th(IV), and U(VI) supplied as the nitrates (5 μg of each metal, 5 μL of each applied to paper), separated as above, and visualized on air-dried papers (30) by spraying with 0.15% arsenazo III (w/v, aqueous (28, 30)). The R_f positions of each were determined by preliminary tests using one metal only, and then the metals were tested in pairs and finally in a three metal mixture (30). The R_f position of each was the same throughout (30).

Visualization and Quantification of ²³⁹Np, ²³⁷Np, and ²³³Pa. ²³⁹Np in cell-free supernatants obtained after centrifugation (20 min at 13 000g) was separated using paper

chromatography and visualized by emission of γ radiation using a phosphorimager-based technique (9). Air dried chromatograms were wrapped in plastic laboratory film and exposed to a storage phosphor screen (Molecular Dynamics, Sevenoaks, Kent, U.K.). The spots of radioactivity were visualized using a phosphorImager (Molecular Dynamics) after 48 h of exposure. In experiments where ²³⁷Np was used without ²³⁹Np γ tracer (the α-activity of ²³⁷Np does not penetrate the clingfilm used to wrap the samples and therefore is not quantifiable by the phosphorImager technique) the radionuclide was quantified directly in cell-free supernatants using a scintillation counter with α, β discrimination. The chromatography paper, containing separated radionuclides (²³⁷Np and ²³³Pa daughter), was cut into 1 cm sections, and each section was added to 10 mL “Ultima Gold” scintillation cocktail (Packard Instrument Company, Meriden CT 06450, U.S.A.) and counted using a Tri-Carb 2700TR Liquid Scintillation Analyzer (Packard Instrument Company). Residual ²³⁷Np in solution was calculated by adding 50 μL of aqueous sample to 10 mL of scintillation cocktail. Uptake of ²³⁷Np by the cells (no attempt was made to discriminate between precipitation, biosorption or bioaccumulation) was calculated by comparing disintegrations min⁻¹ in supernatants to disintegrations min⁻¹ in cultures containing cells. ²³⁷Np and ²³³Pa were discriminated by counting in the α and β windows, respectively.

Results and Discussion

Bioreduction tests utilized *Shewanella putrefaciens*, chosen on the basis of its ability to reduce U(VI) (7, 8) Fe(III) (13), and Tc(VII) (9). The studies reported here were facilitated by the adaptation of a technique (“phosphorImager”) to visualize β- and γ-species spatially separated chromatographically and imaged via a storage phosphor screen (9). Preliminary tests used a surrogate metal mixture to establish likely R_f values for possible product valence species (III, IV, VI: Figure 1A) by direct staining of separated spots using arsenazo III. There is no convenient surrogate for Np(V) so tests were done using Np directly. A solution of ²³⁷Np, freshly prepared by ion-exchange chromatography and free from co-contaminants and daughter products (M. John: personal communication) was donated by BNFL and gave no signal (Figure 1B) because the phosphorimager technique does not detect α-activity (above). A solution of ²³⁷Np obtained from a commercial source (i.e. containing ²³³Pa) gave no signal at the high-valence positions (Figure 1C; cf.1A) but produced a nonmigrating radioactive spot, attributed to the active, β-emitting daughter ²³³protactinium(V). Alone among the pentavalent actinides, Pa(V) hydroxylates to give insoluble, colloidal hydroxide species (5, 31), distinguishing it from Np(V) which is highly soluble (4, 5). The insoluble ²³³Pa did not move from the origin in the chromatographic separation; therefore the behavior of ²³⁷Np could be followed in the presence of ²³³Pa. To confirm the identity of Np in solution purified ²³⁷Np (no other species were present and the α-activity is not detected: Figure 1B) was “spiked” with ²³⁹Np (β,γ-emitter), giving a smeared streak by phosphorImager detection (Figure 1D), between the (IV) and (VI) positions identified in 1A and possibly corresponding to a redox equilibrium of Np(VI), Np(V), and Np(IV) but mainly Np(V). It is well-known from the literature that the Np(V) species is the most stable in solution. As a check the commercially obtained ²³⁷Np/²³³Pa was separated chromatographically (the α-emitting ²³⁷Np was not seen by the phosphorImager technique), and instead cut sections were scintillation-counted with α,β-discrimination. This showed clearly the α and β peaks of the respective Np/Pa components (Figure 1E). The short half-life of the ²³⁹Np tracer (2.35 days) precluded its routine use; further tests used commercially obtained ²³⁷Np since good chromatographic separation was obtained between the parent

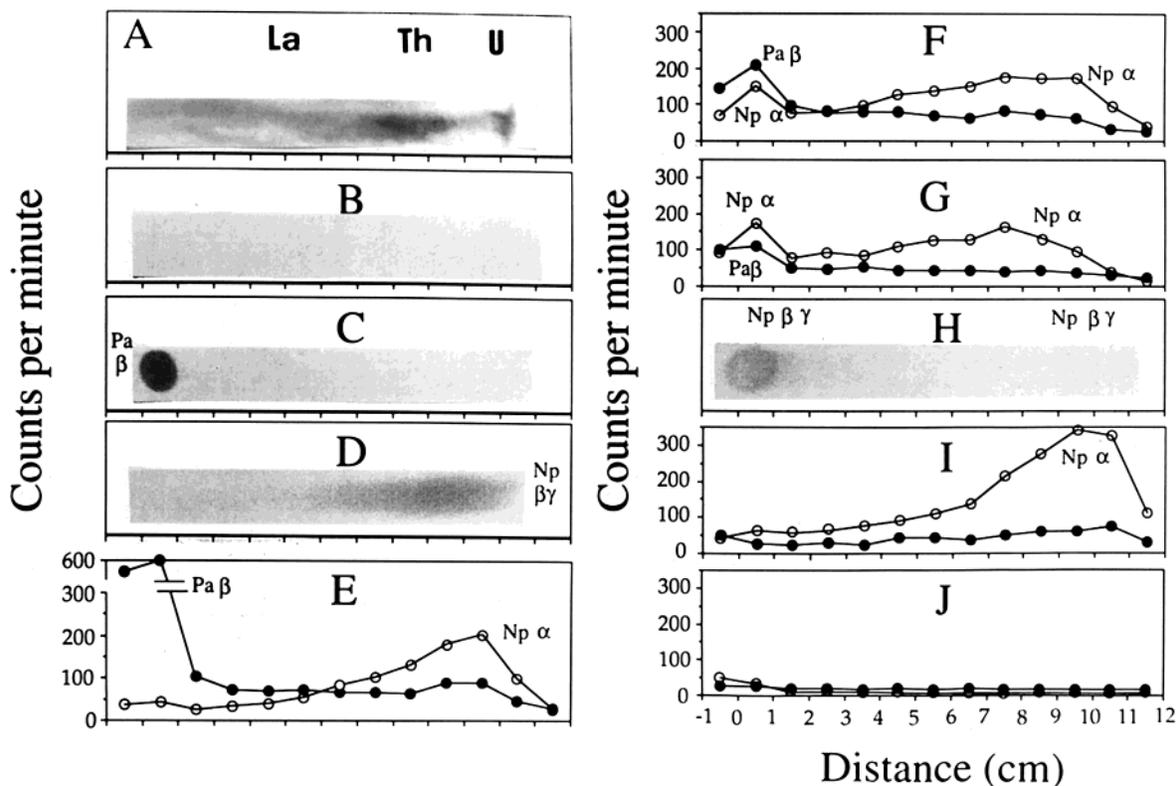


FIGURE 1. The use of microorganisms for the remediation of Np solutions. (A) Chromatographic separation of surrogate metal species in mixed valence solution. A mixture of La(III), Th(IV), and U(VI) (see Materials and Methods) was separated, and the R_f positions of each valence species were visualized using arsenazo III. (B,C) Chromatographic separation of species in solutions of ^{237}Np (separated as in (A)). Detection (phosphorImager) was sensitive to β and γ -emissions via a storage phosphor screen (9). This confirms that the ^{237}Np (α -emitter) is not detected by the technique and that the solution was purified of ^{233}Pa (β -emitter) with testing as follows: (B) Chromatographic separation/phosphorImager detection of a solution ($2\ \mu\text{M}$; $10\ \mu\text{L}$) of ^{237}Np (α -emitter: not found by phosphorImager) separated from daughter products by ion exchange and supplied as purified solution by BNFL. (C) Chromatographic separation/phosphorImager detection of a commercially available solution of ^{237}Np ($100\ \mu\text{M}$) containing ^{237}Np (α -emitting; phosphorImager "silent") and β -emitting daughter ^{233}Pa . Details as in (B). The concentration was increased in order to obtain the R_f position of the ^{233}Pa and to show that ^{237}Np is not detectable by the technique. (D) Chromatographic separation and detection of Np species in a clean background of ^{237}Np (shown in (B)) supplemented with ^{239}Np (β,γ -emitter; $2\ \text{pM}$) and visualized using the phosphorImager following chromatographic separation as in (A). This shows the R_f position of the Np species in the starting solution. (E) Chromatographic separation and detection (by counting) of species within commercially obtained ^{237}Np (α -emitter; $100\ \mu\text{M}$) and decay product ^{233}Pa (β -emitter) separated chromatographically (as (A)) and detected using scintillation counting. \circ : distribution of α -activity (^{237}Np). Note similar R_f position to (D). \bullet : distribution of β -activity (^{233}Pa). Note similar R_f position to that in (C). (F) Nuclide distribution following chemical reduction of commercially obtained ^{237}Np using ascorbic acid (1% wt/vol aqueous final concentration), separated (as (A)) and counted (as (E)). Note that the R_f position has shifted from mid-way between the Th(IV) and U(VI) calibration positions (E), cf. (A) to the position of Th(IV) (F), cf. (A). Only 12% of the ^{237}Np ($100\ \mu\text{M}$) was removed from solution by centrifugation prior to analysis. (G) Hydrogen-dependent bioreduction of commercially available ^{237}Np (mixture of ^{237}Np and ^{233}Pa : see (E)) with *S. putrefaciens* (see Materials and Methods). Note that biosorption of ^{233}Pa was observed; this nuclide sorbs onto most surfaces (37). Little Np is apparent between the R_f positions of Th(IV) and U(IV) as shown in (A) and the Np position peaks at the R_f position shown for Th(IV) in (A). Only 25% of the ^{237}Np ($100\ \mu\text{M}$) was removed with the biomass by centrifugation prior to analysis. (H) Bioreduction of ^{239}Np in carbonate buffer. *S. putrefaciens* was prepared (as in (G)) and resuspended in 30 mM carbonate buffer (pH 7.5). The samples were supplemented with "cleaned" ^{237}Np (bulk isotope: to $2\ \mu\text{M}$) and ^{239}Np (spike: to $2\ \text{pM}$). ^{239}Np species (β,γ -emission) were separated as in (A) and visualized as in (D). (I) Bioaccumulation of ^{233}Pa and ^{237}Np by *Citrobacter* sp. Chromatographic separation as in (A). Note loss of ^{233}Pa from solution but negligible bioreduction or removal of Np. (J) Removal of ^{233}Pa and ^{237}Np by the concerted action of the two microorganisms. Suspensions of both organisms were under H_2 as in (G) and were supplemented with 5 mM glycerol 2-phosphate and 100 mM ammonium acetate as in (I). Under these conditions, 95% of the ^{237}Np was removed by centrifugation prior to analysis.

nuclide and the daughter element.

Chemical reduction of Np(V) using ascorbic acid gave a loss of activity in the R_f position between the Th(IV) and U(VI) calibration positions and a migration to a lower R_f value corresponding to that of Th(IV) (Figure 1F) and also a new α -peak overlaying the β -peak of ^{233}Pa , at the expense of ^{237}Np (V) (1F). This suggested reduction of Np(V) to colloidal, nonmigrating hydroxylated species of Np(IV) via a reduced species. A portion of the Np did not correspond unequivocally to the position of Th(IV) or La(III) (cf. Figure 1A), but hydroxylation of the higher actinides and formation of colloidal material is more extensive than for Th(IV) (5),

distinguishing the latter from Np (IV). Treatment of ^{237}Np with *Shewanella putrefaciens* gave a similar species distribution (1G) to that obtained using ascorbic acid (Figure 1F) suggesting bioreduction of Np(V) to Np(IV). However in both cases the reduction was incomplete. This is probably because without removal of the reduced Np(IV) from solution reestablishment of the disproportionation equilibrium was occurring in parallel to the reduction reactions.

The experiment was also done with $^{237/239}\text{Np}$, in carbonate buffer and at a lower Np concentration to mimic more closely the situation of a natural water. Little β,γ activity was detected

at the R_f positions corresponding to mobile valence (VI), (V), (IV), or (III) species; the recovered ^{239}Np was at the position corresponding to that of the ascorbate-reduced, nonmobile ^{237}Np (Figure 1H; cf. 1D). This suggests that in carbonate buffer and at low concentrations the soluble Np species are removed from solution, but whether this was attributable to biosorption onto the biomass was not tested.

In accordance with previous findings using Tc(VII) (9), little Np was removed from a 100 μM solution by *S. putrefaciens* or following chemical reduction (25% and 12% removal of the input Np, respectively, corresponding to 85% and 88% of the Np remaining in solution), i.e., little was removed using bioreduction alone. This is in contrast to the bioreduction of U(VI), where U(IV) was precipitated as insoluble UO_2 (7, 8). Tetravalent actinides are insoluble at pH 7 due to extensive hydroxylation and oxide formation (4, 5, 24). In contrast to the precipitation of UO_2 , the conversion of hydroxylated Pu(IV) to the insoluble oxide (PuO_2) is slow (26, 32), i.e., Pu(IV) does not precipitate immediately, and it is likely that the hydroxylated neptunium(IV) is, similarly, a soluble, nascent microcolloid which does not migrate chromatographically and which does not precipitate onto biomass *per se*, especially in the presence of 100 mM ammonium acetate (above). Hence, the removal of Np from solution by ascorbic acid treatment or bioreduction alone is likely to be low. These effects are difficult to confirm experimentally since the speciation of Np in solution is complex (14) and the physicochemical contribution of the bacterial cell surface (microenvironmental effects) is difficult to assess, given that little is known of the reactive groups on the extracellular polymeric matrix of the *Shewanella* sp. or, indeed, the way in which these might change according to the growth conditions of the organism. It can be concluded, however, that *S. putrefaciens* removed little Np from solution (Figure 1G and see above).

In separate experiments *Citrobacter* sp. did not remove native (nonreduced) $^{237}\text{Np(V)}$ (Figure 1I) in the presence of glycerol 2-phosphate, under which conditions Th(IV) and Pu(IV) were removed via phosphate precipitation (11, 33). This is a simple hydrolysis reaction (cleavage of glycerol 2-phosphate as a donor of inorganic phosphate at the cell surface); no input of energy is required, and the presence of hydrogen is not relevant to the phosphate precipitation reaction. In the present system the *Citrobacter* sp. was incorporated to scavenge specifically the Np(IV) in the presence of Np(V) by analogy with its ability to remove Pu(IV) from solution as plutonium phosphate (11, 33). Although the removal of Np was negligible, ^{233}Pa was removed (Figure 1I), in accordance with the high insolubility of protactinium phosphate (5, 31). Without reduction Np(V) was retained at the appropriate R_f position between that of Th(IV) and U(VI) in accordance with the inability of *Citrobacter* sp. to remove Np(V) *per se* (11) (see Figure 1I). In the presence of *S. putrefaciens* 95% of the Np was removed from solution by *Citrobacter* sp. (Figure 1J), as anticipated from the low solubility products of the tetravalent actinide phosphates (4) and in accordance with previous studies using Th(IV) and Pu(IV) (11, 33). An identical result was obtained using *Citrobacter* sp. challenged with ascorbic acid-reduced Np, suggesting strongly that the role of the *Shewanella* was to reduce Np(V) to Np(IV), in which form it becomes amenable to the *Citrobacter*-mediated phosphate bioprecipitation.

Thus, we have demonstrated the removal of Np(V) using a coupled system, and the results suggest that this is via a concerted bioreduction and phosphate biomineralization system. Previous attempts to accumulate Np(V) onto biomass gave only negligible uptake (11, 33–35), as anticipated from the chemistry of NpO_2^+ . It was postulated previously (2, 6) that hydrogenase-mediated bioreduction of actinides would be a distinct possibility. The role of hydrogenase in the

bioreduction of $^{99}\text{Tc(VII)}$ by *Escherichia coli* (36) and *Desulfovibrio desulfuricans* (37) is now established, using H_2 as the electron donor. The potential harnessing of growth-decoupled hydrogen-dependent metal reductase and phosphatase activities of two organisms makes an integrated, "clean" technique for bioremediation of neptunium wastes uniquely possible. The use of hydrogen as the feedstock is noteworthy, since this introduces no additional organic load, and no waste product is generated from H_2 oxidation. Individually, *Citrobacter*, *E. coli*, and *D. desulfuricans* bioreactors have been used continuously over several weeks in the removal of metal as phosphate (11, 19) and in the bioreduction of $^{99}\text{Tc(VII)}$ (38, 39), respectively, and there is no reason why the coupled system should not be similarly stable. Provision of H_2 from a bottled supply could be uneconomic or present a potential hazard. However in other tests we have shown that bioreduction of Pd^{2+} to Pd^0 by *D. desulfuricans* is supported by electrochemically generated H_2 which can be supplied on demand for continuous metal removal using immobilized cells in an electrobioreactor (40), and such electrobioreactors have been shown to be stable in operation over long periods for continuous metals recovery.

This preliminary study illustrates the potential for the biotechnological removal of Np(V), which is not easily achieved chemically. Continuous-flow bioreactors for actinide removal by *Citrobacter* sp. (11, 19) and metal reduction by *E. coli* and sulfate-reducing bacteria (37–39) are already established. It is anticipated that future studies will seek to establish the nature of the solution chemistry during Np(V) reduction, but these tests should be carried out in realistic waste solutions, since these will contain other species which will influence the chemistry of the Np. As an example, many wastes contain chelating agents (3, 41) and may also be at high or low pH, and a realistic assessment should be made in an actual waste solution. It is hoped that this preliminary demonstration will stimulate further study of the potential role of bioremediation in Np removal from real wastes *in situ* or by using "pump and treat" methods.

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