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## Reduction and Precipitation of Neptunium(V) by Sulfate-Reducing Bacteria

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### Abstract

Migration of neptunium, as  $\text{NpO}_2^+$ , has been identified as a potentially important pathway for actinide release at nuclear waste repositories and existing sites of subsurface contamination. Reduction of  $\text{Np(V)}$  to  $\text{Np(IV)}$  will likely reduce its solubility, resulting in lowered subsurface migration. The ability of sulfate-reducing bacteria (SRB) to utilize  $\text{Np(V)}$  as an electron acceptor was investigated, because these bacteria are active in many anaerobic aquifers and are known to facilitate the reduction of metals and radionuclides. Pure and mixed cultures of SRB were able to precipitate neptunium during utilization of pyruvate, lactate, and hydrogen as electron donors in the presence and absence of sulfate. The neptunium in the precipitate was identified as  $\text{Np(IV)}$  using X-ray absorption near edge spectroscopy (XANES) analysis. In mixed-culture studies, the addition of hydrogen to consortia grown by pyruvate fermentation stimulated neptunium reduction and precipitation. Experiments with pure cultures of *Desulfovibrio vulgaris*, growing by lactate fermentation in the absence of sulfate or by sulfate reduction, confirm that the organism is active in neptunium reduction and precipitation. Based on our results, the activity of SRB in the subsurface may have a significant, and potentially beneficial, impact on actinide mobility by reducing neptunium solubility.

### Introduction

The goal of this research is to determine if and how the metabolic activity of sulfate-reducing bacteria (SRB) decreases the solubility of neptunium in the subsurface. A likely mechanism is the SRB-facilitated bioreduction of  $\text{Np(V)}$  to  $\text{Np(IV)}$ , leading to the precipitation of  $\text{Np(IV)}$  phases. The impact of this research is potentially far-reaching, because SRB are ubiquitous to the anaerobic environments [1-3] that are present in most situations where contaminant migration is of concern.

Sulfate reducing bacteria (SRB) are quite diverse in their metabolism and metal-reducing capabilities. They reduce  $\text{Fe(III)}$ ,  $\text{U(VI)}$ ,  $\text{Mo(V)}$ ,  $\text{Tc(VII)}$ , and  $\text{Cr(VI)}$ ; some of these metals may be used by SRB as electron acceptors for energy generation [2, 4-10]. The metabolic versatility of SRB enables them to survive and function in diverse environments. When sulfate is present, SRB can couple the oxidation of simple organic substrates or hydrogen to sulfate reduction. Conversely, in the absence of sulfate, SRB can ferment simple organic compounds like lactate and pyruvate to acetate and

molecular hydrogen. This ability to function as hydrogen producers or consumers is the reason that SRB are an integral part of most anaerobic consortia [1, 11-15].

The potential immobilization of actinides under reducing conditions has been described by numerous authors [for example, see 16-23]. Figure 1 shows the relationship between reduction potentials typical of microbiological activity and actinide oxidation states [24]. For neptunium, Np(V) is predicted to be the dominant oxidation state in oxygenated waters. Under most anaerobic conditions (e.g., the fermentative and sulfate-reducing biological systems investigated herein), however, the thermodynamically stable oxidation state is Np(IV).

A link between SRB activity and the reduction of Np(V) is also suggested by a number of experimental results and observations reported in the literature. The predominance of Np(IV) in some anaerobic environments has been reported [25-31] and associated with sulfate-reducing systems [26, 27]. In cases where the redox potential or dominant biological process in the system was reliably determined, the predicted redox speciation of neptunium agreed well with experimental data. Hakanen and Lindberg [31] found that Np(IV) was the major oxidation state in pH 9 groundwater with a measured  $E_h$  of -290 mV. Additionally, we have shown [27] that Np(IV) is the dominant oxidation state in reducing methanogenic sediments. Lastly, neptunium sorption in anaerobic systems increases by several orders of magnitude, presumably by reduction of Np(V) to Np(IV) [25, 30].

## Materials and Methods

### Culture Growth Conditions – Pure Cultures

A pure culture of the sulfate-reducing bacterium *Desulfovibrio vulgaris* subspecies *vulgaris* (ATCC 29579) was obtained from frozen stock maintained in the Microbial Ecology Laboratory at Northwestern University. The organism was cultured in a bicarbonate-buffered medium described previously [14, 32, 33]. The techniques for growth medium preparation and culture maintenance were adapted from Brusseau [33] and Noguera et al. [14]. The pH was adjusted to 7.2 with sodium

hydroxide. The medium was transferred under  $N_2/CO_2$  into serum bottles, and the bottles were sealed, flushed, pressurized to 2 psig, and autoclaved for 20 min. Concentrated stock solutions (750 mM) of donors (Na-lactate, -acetate) and acceptor ( $Na_2SO_4$ ) were prepared, added to sealed, sterile serum bottles, and sterilized by autoclaving. The stock solutions were regularly purged with sterile, high-purity He gas to maintain anoxic conditions and were transferred to culture bottles via a needle syringe. The medium was reduced by adding 0.06 g/l  $Na_2S$  4 hours prior to inoculation, and cultures were maintained by transfer of a 5% inoculum into fresh medium. Maintenance cultures were incubated at 30°C and shaken at 150 rpm. Culture growth was followed by measuring the absorption at 600 nm or by determining dry weight.

#### Culture Growth Conditions – Mixed Cultures

The microbial culture used for the mixed-culture studies was obtained from the Environmental Research Division at Argonne National Laboratory. The consortium was isolated previously from creek sediment [34]. Characterization of the anaerobic, sulfate-reducing consortium identified four *Desulfovibrio* spp., including *D. desulfuricans* A, *D. desulfuricans* B, *D. gigas*, and a strain closely resembling *D. vulgaris* [34, 35]. No characterization was performed for other anaerobic, fermentative, or facultative organisms in the consortium.

For these studies, the consortium was grown at 25°C in sealed 70-ml serum bottles. The anaerobic techniques described above were used. A growth medium was prepared as described previously [34]. The pH of the medium was adjusted to 6.4 with NaOH, sterilized through a 0.2- $\mu$ m filter, and dispensed into sealed, sterile serum bottles by using a needle syringe. The bottles were purged for 20 min with sterile high-purity nitrogen or  $N_2/CO_2$  (72/28%) gas mixture. Due to the higher carbonic acid concentration in equilibrium with the  $N_2/CO_2$  mixture, the final pH of the medium was 5.4 when this gas was used. Cultures were maintained by needle-syringe transfer of a 5% inoculum from actively growing cultures to fresh medium.

## Batch Experiments

Batch experiments were conducted in serum bottles sealed with thick rubber stoppers. Except for adjustment of electron donors and acceptor, the experimental medium for each culture was identical to the two growth media described above. After addition of the electron donors and/or acceptor, the bottles were purged with sterile N<sub>2</sub>/CO<sub>2</sub> for 20 min. Sterile hydrogen gas was added with a needle syringe. Electron donors and acceptor were transferred to the experimental bottles by sterile and anaerobic techniques. The pH was measured by a combination glass electrode and qualitatively checked with pH paper. Experiments were initiated by sterile, needle-syringe transfer of a 5% inoculum from an actively growing culture. All experimental and maintenance cultures were grown in an incubating shaker at the temperatures stated earlier and at 150 rpm.

## Preparation of Sterile, Anoxic Np(V) Stock

A stock solution of Np(V) was prepared by column separation (Biorad AG-50) of <sup>237</sup>Np from Pu and Pa. The neptunium was taken to dryness and re-dissolved in 2 ml of 0.01 M HCl, resulting in a solution of mixed Np(V/VI) oxidation state. This solution was again diluted with 15 ml of 0.01 M HCl and electrolytically reduced [36] for 30 min (-1.0 V potential; 35 mA current; 0.5 V current breakpoint). The oxidation state purity was determined by VIS-NIR spectroscopy [37]. The actinide isotopic purity was determined by comparison of alpha scintillation counting results, ICP-MS analysis, and the absorption spectra. Isotopic and oxidation state purity was greater than 95%. The stock solution concentration was 2.0 mM. Approximately 8 ml of the Np(V) stock was sterilized by filtration (0.2  $\mu$ m) and transferred by needle syringe into a sterile, 10-ml serum bottle with a rubber stopper. The stock Np(V) solution was regularly purged with sterile, high-purity Ar gas to maintain anoxic conditions. Aliquots of Np(V) were added to experimental bottles by sterile, anoxic needle syringes.

## Sampling and Analytical Methods

Experimental bottles were sampled periodically with a needle syringe. Biomass growth was monitored by optical density at 600 nm or dry weight measurement. Neptunium solubility was determined by comparison of scintillation counts (Packard) from equal volumes of 0.2- $\mu$ m filtered and unfiltered aliquots of solution. Bottles were first sampled with a needle syringe, and a portion of the sample was transferred to a weigh dish. The needle was then removed from the syringe and replaced with a 0.2- $\mu$ m syringe filter. The remainder of the solution was filtered and transferred to a second weigh dish. In this way, the samples were filtered before being exposed to air, minimizing the potential for oxidation of any precipitates. Known volumes were drawn from the weigh dishes with a pipette and added to a scintillation cocktail (Ultima Gold) for subsequent counting.

X-ray absorption near-edge spectroscopy (XANES) analyses [27, 38-41] were performed on the MR-CAT undulator beamline at the Advanced Photon Source (APS) at Argonne National Laboratory. Samples were prepared in a nitrogen glovebox by gravity centrifugation (6000 rpm and 30 min) in 10-ml centrifuge tubes. Following centrifugation, the biomass pellet was encapsulated in polystyrene plastic. After encapsulation, the samples were removed from the glovebox and mounted for XANES analysis. Neptunium standards of various oxidation states were prepared by recovering solids from the following syntheses: Np(IV)F<sub>4</sub> by addition of excess fluoride to Np(IV); Np(V)NaCO<sub>3</sub> by titration of Np(V) stock with 0.1 M sodium carbonate to pH 7; and Np(VI)-phosphate by addition of excess phosphate to Np(VI) stock and adjustment of pH to 8.

## Results and Discussion

### Neptunium Precipitation by Mixed Anaerobic Cultures

A series of experiments was performed to investigate the ability of non-growing cells (supplied only electron donors without other nutrients) to utilize Np(V) as an electron acceptor. To avoid substrate carryover, the inoculum for these experiments was harvested in log phase of growth and rinsed

according to the procedure described previously [9]. The experimental bottles were prepared as described previously for experiments studying U(VI) reduction by SRB [9]. Rinsed cells showed normal growth in the full medium. Under non-growing conditions, the cells did not precipitate any neptunium when either H<sub>2</sub> or formate was supplied as an electron donor. The experiments were repeated in sterile, high-purity water and 20-mM phosphate buffer, giving no neptunium precipitation in all cases. Complexation of neptunium in the phosphate buffer would have mitigated toxicity effects from the aquo NpO<sub>2</sub><sup>+</sup> ion [42]. Thus, non-growing cells did not precipitate neptunium under all conditions investigated.

Additional experiments were performed to investigate potential neptunium precipitation by growing cultures. Neptunium and supplemental electron-donor additions were made to cultures grown on 30 mM pyruvate and 20 mM sulfate for 4 days; the experimental setup is detailed in Table 1. Figure 2 shows the results from this experiment. In all cases, neptunium was precipitated, but at different rates. Complete replacement of pyruvate with H<sub>2</sub> accelerated neptunium precipitation. However, addition of H<sub>2</sub> and pyruvate together slowed neptunium precipitation, as compared to the addition of either H<sub>2</sub> or pyruvate alone. The difference in precipitation rate was not caused by toxicity of neptunium, because the sample that showed the highest rate, which received only hydrogen as a supplemental electron donor, also had the highest neptunium concentration ( $\sim 0.9 \times 10^{-5}$  M total neptunium). At the end of the experiment (18 days), the pH of all samples was checked to verify that the precipitation was not caused by a pH-induced shift in neptunium chemical speciation. The pH of samples 1 and 2 was approximately  $6.1 \pm 0.2$ ; the pH of samples 3 and 4 remained about  $5.9 \pm 0.2$ . Thus, the chemical speciation was not affected significantly. Although sulfate was present in the growth medium in these experiments, no sulfate reduction was detected during the experiment (sulfate concentration measured by ion chromatography, data not shown). This result indicates that the primary metabolism of the culture was pyruvate fermentation, not sulfate reduction.

The precipitation results shown in Fig. 2 are typical of the trends observed in similar mixed-culture experiments. Cultures receiving the highest pyruvate additions showed the lowest rate of neptunium precipitation (data not shown). We attribute the difference in precipitation rates to complexation of Np(IV) by intermediates generated during pyruvate degradation; when more pyruvate was added, more intermediates were generated [43]. The role of hydrogen in facilitating neptunium precipitation is still not clear, however. Perhaps the availability of hydrogen increased the rate of intermediate degradation or selected for organisms capable of neptunium reduction [43].

### Neptunium Precipitation by Pure Cultures

Additional experiments were conducted to determine the fate of neptunium in systems when *D. vulgaris* grew using lactate or hydrogen as electron-donor substrates with sulfate as an electron acceptor, or when the organism grew by lactate fermentation in the absence of sulfate. Table 2 details the setup of these investigations. For the first three experiments, Np(V) was added to cultures in the log phase of growth. For the fourth experiment, Np(V) was added to cultures inhibited by hydrogen produced during lactate fermentation. Figure 3 shows that neptunium precipitated in all cases, although the initial (24-hr) rate of precipitation varied depending on the metabolic activity of the organism. The initial rate of neptunium precipitation was highest when cultures grew with hydrogen or lactate as an electron donor with or without sulfate as an electron acceptor. The 24-hr neptunium precipitation was slowest when post-log phase cultures grew by lactate fermentation.

Hydrogen is an essential part of lactate metabolism by *D. vulgaris*, even when sulfate is present as an electron acceptor [34]. For example, Noguera et al. [14] estimated that about 50% of the electrons obtained from lactate oxidation by *D. vulgaris* were cycled through hydrogen production and consumption. In the absence of external electron acceptors, *D. vulgaris* couples lactate oxidation to hydrogen production during fermentation. Thus, hydrogen is available to the organism under all growth conditions. For this reason, the precipitation results shown in Fig. 3 may support the observation from

mixed-culture experiments that hydrogen plays a role in neptunium precipitation. Neptunium precipitation was fastest during periods of rapid growth when the rate of hydrogen processing by the organism was highest; neptunium precipitation was slowest when the growth of the organism was inhibited by hydrogen [43]. However, we cannot at this time rule out the possibility that the rate of neptunium precipitation depended on the growth rate of *D. vulgaris*. These hypotheses will be evaluated in future work.

To directly establish the neptunium oxidation state in the precipitate, XANES analysis was conducted on neptunium solids recovered from the two fermentation experiments. Figure 4 shows XANES spectra of neptunium solids from the two lactate fermentation experiments compared to spectra obtained from Np(IV) and Np(V) solid standards. Because recent evidence suggests that the difference in XANES edge position is less than 1 eV between Np(IV) and Np(V) [27], the neptunium oxidation state is qualitatively determined by the presence or absence of the near-edge shoulder associated with the linear dioxo-cation structure [44]. In Fig. 4, the edge positions of the unknown spectra indicate that neptunium is present as Np(IV) or Np(V), not Np(III) or Np(VI). Because the spectra from precipitated neptunium in Fig. 4 lack the near-edge feature associated with the neptunyl structure, the results support the conclusion that the precipitate is an Np(IV) solid.

## Conclusions

The results of this research show that a mixed anaerobic consortium and pure cultures of *D. vulgaris* actively precipitated neptunium from solution. The XANES analysis of neptunium in the precipitate supported the view that neptunium was reduced to Np(IV). This work is the first documented report of microbially driven Np(V) reduction. For mixed cultures, neptunium precipitation was most rapid when cultures were supplemented with hydrogen as the electron donor in lieu of pyruvate. For pure cultures, the rate of precipitation depended on the metabolic activity of *D. vulgaris*. Neptunium precipitation was most rapid when hydrogen or lactate were supplied as electron donors.

Precipitation of neptunium did not require the addition of sulfate as an electron acceptor, as cultures growing by lactate and pyruvate fermentation were able to drive neptunium precipitation.

The discovery that *D. vulgaris* and other anaerobic bacteria can drive reduction of Np(V) is an important result that has implications for neptunium mobility in anaerobic environments. Because SRB are common in anaerobic systems, if their ability to stimulate neptunium reduction is widespread, neptunium migration might be retarded significantly when reduction occurs. However, more research is needed to determine the mechanism for neptunium reduction by SRB and other microorganisms and to investigate the stability of bioprecipitated neptunium in natural environments.

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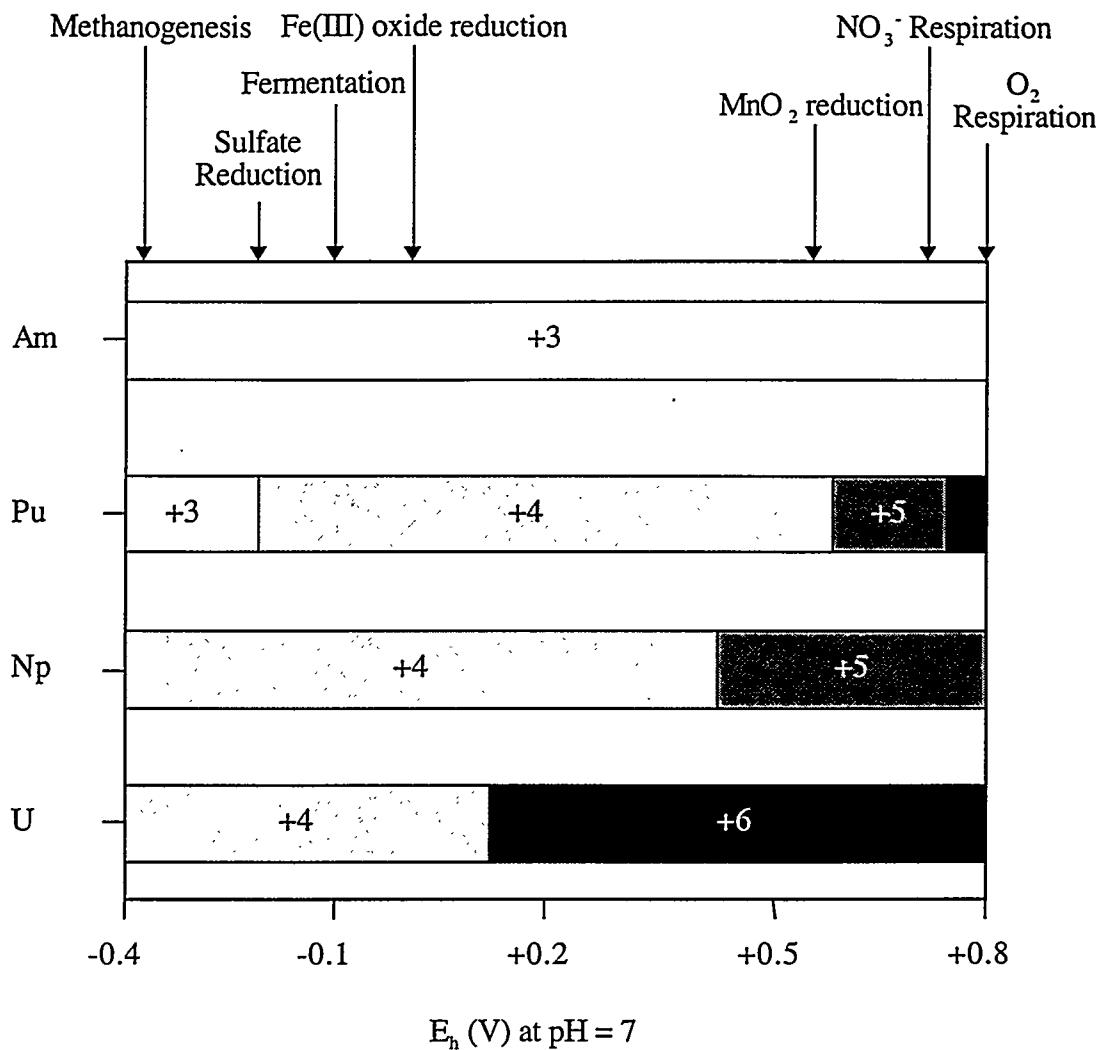
This work was partially funded by Argonne National Laboratory directed research funds to investigate actinide speciation in environmental systems and by a Cabell Fellowship from the McCormick School of Engineering and Applied Science at Northwestern University. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Basic Energy Sciences, Office of Energy Research, under Contract No. W-31-109-Eng-38. The MR-CAT at the APS is supported by its member institutions.

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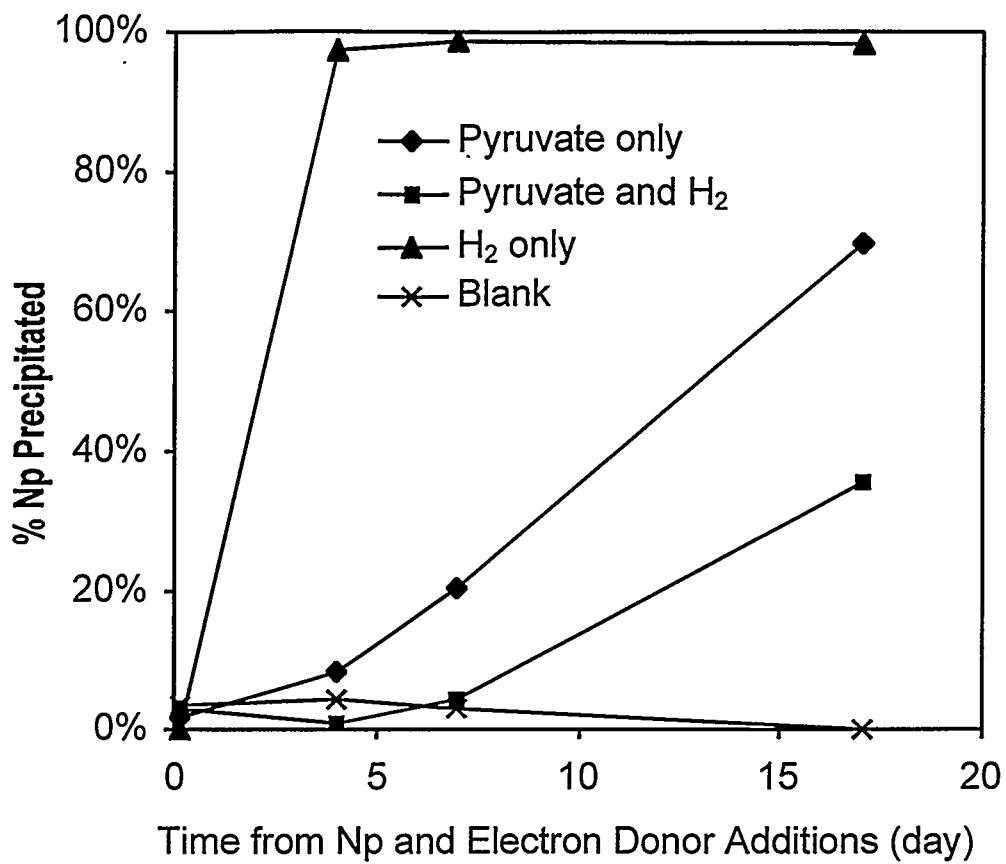
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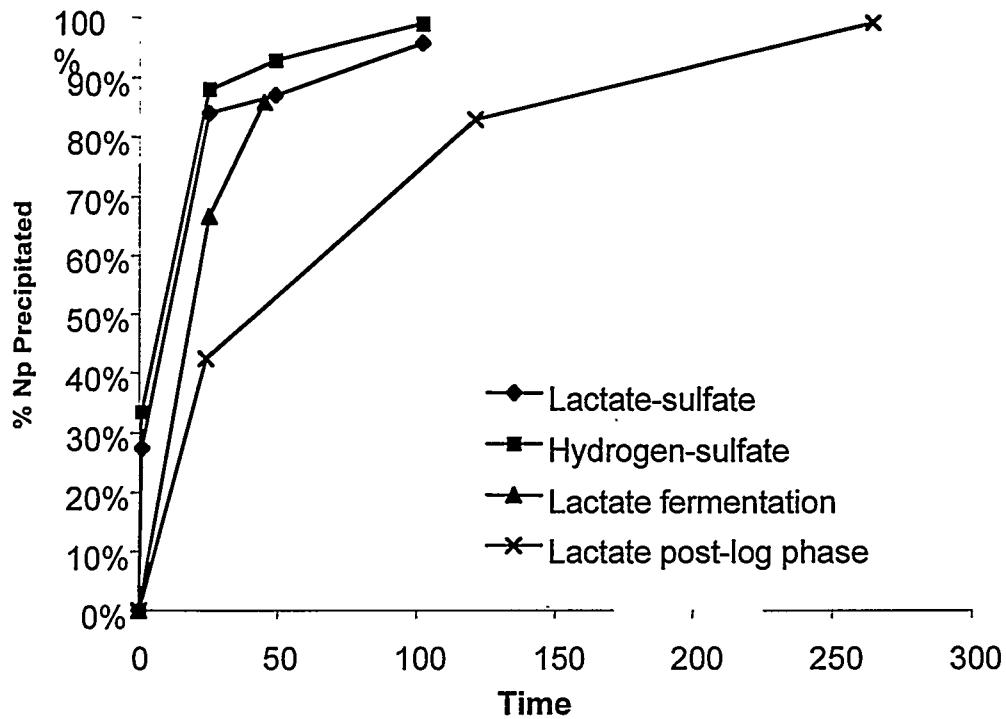
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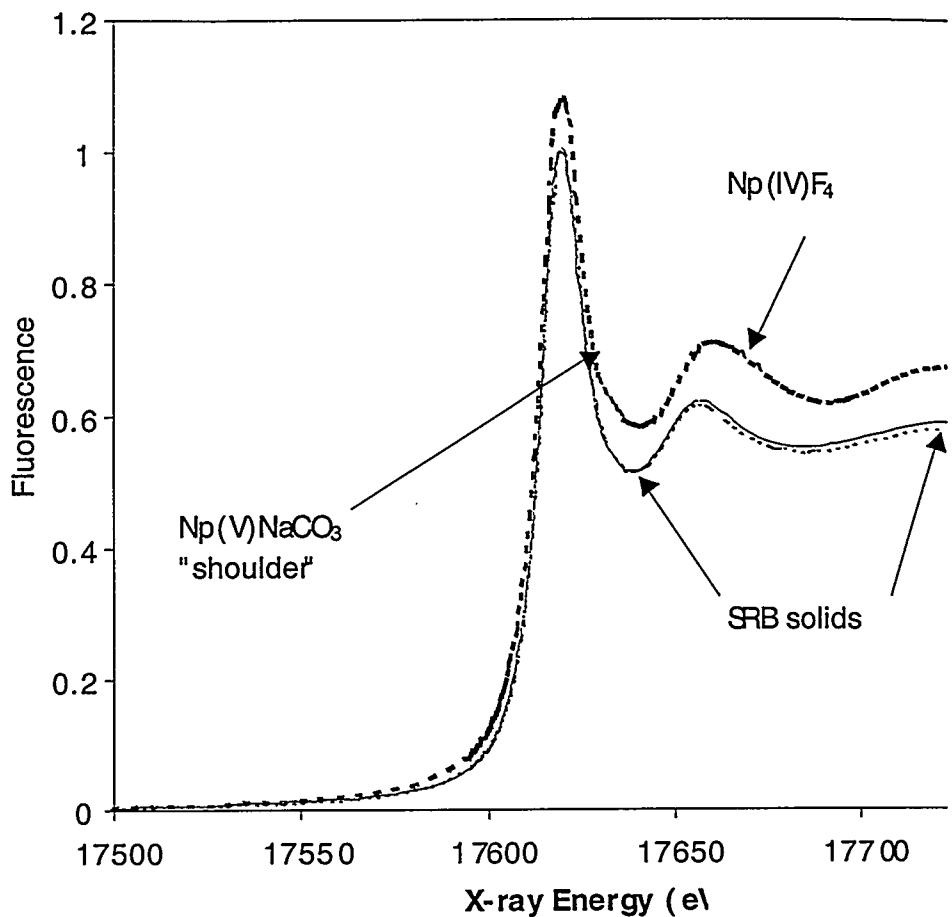
**Figure 1.** Expected dominant oxidation states of the actinides as a function of standard reduction potential in pH 7 water at equilibrium with atmospheric CO<sub>2</sub>. The expected redox potentials associated with common microbial electron acceptor couples are shown schematically at the top of the figure. Adapted from Silva and Nitsche [43] and Stumm and Morgan [45].



**Figure 2. Fate of neptunium during growth of the mixed anaerobic consortium. Addition of hydrogen accelerated Np precipitation. Addition of hydrogen and pyruvate caused the lowest precipitation rate.**



**Figure 3.** Neptunium precipitation by pure cultures of the SRB *Desulfovibrio vulgaris*. Precipitation was most rapid when the organism was in the log phase of growth and was provided sulfate as an electron acceptor. *D. vulgaris* also catalyzed neptunium precipitation when growing by lactate fermentation in the absence of sulfate. The rate of precipitation was lowest when neptunium was added to cultures in the post-log phase of growth.



**Figure 4.** Comparison of XANES spectra from neptunium precipitated during lactate fermentation to  $\text{Np(IV)}$  and  $\text{Np(V)}$  solid standards. The bio-precipitated neptunium spectra lacks the near-edge shoulder associated with  $\text{Np(V)}$ , indicating that  $\text{Np(V)}$  was reduced to  $\text{Np(IV)}$  during lactate fermentation.

**Table 1 – Electron Donor Additions for Mixed-Culture Experiments**

| <i>Time of<br/>Addition (days)</i> | <i>Sample Number</i>      |  |                                |       |
|------------------------------------|---------------------------|--|--------------------------------|-------|
|                                    | 1                         | 2  | 3                              | Blank |
| -4                                 | Inoculation<br>30 mM Pyr. | Inoculation<br>30 mM Pyr.                      | Inoculation<br>30 mM Pyr.      | None  |
| 0                                  | Np + 15 mM<br>Pyr.        | Np + 15 mM<br>Pyr. + 12.3<br>mM H <sub>2</sub> | Np + 12.3 mM<br>H <sub>2</sub> | Np    |

**Table 2 – Electron Donors and Acceptor for *Desulfovibrio vulgaris* Experiments**

| <i>Sample</i>          | <i>Electron Donor</i> | <i>Electron Acceptor</i> | <i>Np(V) Added (M)</i> |
|------------------------|-----------------------|--------------------------|------------------------|
| Hydrogen-sulfate       | 8 mM H <sub>2</sub>   | 10 mM sulfate            | 1.3 x 10 <sup>-5</sup> |
| Lactate-sulfate        | 15 mM lactate         | 10 mM sulfate            | 1.3 x 10 <sup>-5</sup> |
| Lactate fermentation   | 12.2 mM lactate       | none                     | 2.0 x 10 <sup>-5</sup> |
| Lactate post-log ferm. | 12.2 mM lactate       | none                     | 2.0 x 10 <sup>-5</sup> |