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# LOS ALAMOS NATIONAL LABORATORY
# CARLSBAD OPERATIONS

## *LCO-ACP-23, REVISION 0*

## Review of the Microbial Colloid Contribution to the WIPP Actinide Source Term

**Effective Date:** 3-15-19 LA-UR-19-22310

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ACP-1903-05-05-01

**History of Revisions**

| Revision Number | Effective Date | Pages Affected | Description of Revision |
|---|---|---|---|
| 0 | 3-15-19 | All | Original Release |

**ACRONYMS**

| | |
|---|---|
| An | actinide |
| BAB | brine A bacteria; mixed culture of extreme halophiles used in CCA tests |
| CAPMIC | parameter used to describe the maximum microbially-associated actinide concentration |
| CCA | Compliance Certification Application |
| CFU | colony-forming unit |
| CRA | Compliance Recertification Application |
| DGR | deep geological repository |
| DPA | dipicolinic acid |
| DSM | Deutsche Sammlung von Mikroorganismen (German microbial culture collection) |
| EDTA | ethylenediamine tetraacetic acid |
| EPS | extracellular polymeric substance(s) |
| ERDA | Energy Research and Development Administration (well 6) |
| GWB | generic weep brine |
| ICP-MS | inductively-coupled plasma mass spectrometry |
| OD | optical density |
| PA | performance assessment |
| PROPMIC | proportionality constant multiplied by the dissolved actinide concentration to calculate the concentration of microbially associated actinide |
| TEM-EDS | transmission electron microscopy with energy dispersive X-ray spectrometry |
| TRU | transuranic |
| US DOE | United States Department of Energy |
| WIPP | Waste Isolation Pilot Plant |

## EXECUTIVE SUMMARY

This report reviews the microbial contribution to the Waste Isolation Pilot Plant (WIPP) colloid model and its effect on the mobile actinide source term to provide a basis for the biocolloid parameters recommended for the 2019 Compliance Recertification Application (CRA). Microbial colloids are one of four types of colloids identified in the model, in addition to intrinsic, mineral and humic colloids. For the original certification process, the parameters PROPMIC and CAPMIC were defined, and experiments were conducted to provide values for those parameters. For Compliance Recertification Application 2014 (CRA; US DOE, 2014), the values of those parameters were changed, based on new data and a new understanding of microbial ecology at the WIPP. Specifically, PROPMIC values—that measure the proportion of the dissolved actinide concentration that is microbially-associated—were lowered, and separate values were provided for archaea and bacteria. Additionally, the derivation of CAPMIC values—the maximum concentration of microbially-associated actinide—was changed from a toxicity-based approach to a biomass-based approach, and separate values were again provided for archaea and bacteria. CAPMIC values were also lowered by this process.

The change in these parameter values for CRA 2014 was called into question, and this has prompted a critical review of all WIPP data, past and present, a new literature survey, and additional experiments to be undertaken. The outcome is presented in this report. Briefly, previous data used for the Compliance Certification Application (CCA; US DOE, 1996) PROPMIC are acceptable but not as relevant as newer data, for the following reasons: 1) bacterial data were used instead of archaeal data, although archaea are more likely to dominate the near-field and 2) actinide speciation was not as well-defined in previous experiments, due to 3) a lack of knowledge at the time about expected WIPP conditions (e.g., pH, brine composition, the presence of organic complexants) and hence the nature of the experimental design. The data used to generate the earlier CAPMIC values are judged to be inconclusive and subject to more uncertainty than the biomass-based CAPMIC presented in CRA 2014.

Of the newer data used for CRA 2014, these also had drawbacks, specifically in the lack of longer term data, although this was not part of the experimental design. Additional experiments conducted for this report have addressed that issue. Some of these new data suggest an even lower contribution of microbial colloids to the overall actinide source term, in that biological influence appears to result in immobilization of actinides via induced precipitation and/or mineralization, rather than mobile colloid formation, and the measured “association” values may only be apparent.

In addition to comparing past and current experimental data, this report addresses additional questions about the predicted biomass loading of the WIPP throughout repository history. These predictions are based on microbial ecology, bioenergetics, and studies from other repository and hypersaline settings. While many questions are not fully answered, there is enough information to support specific recommendations for CRA 2019 and the biocolloid contribution to the actinide source term. The most important conclusions and recommendations of this report are

that 1) the use of archaeal data is still preferred over bacterial data, given the likelihood of archaeal dominance in the microbial population ; 2) the use of the biomass-based CAPMIC increases the realism of the biocolloid model; 3) the use of PROPMIC parameters derived under WIPP-relevant conditions may lower the values but adds realism; and 4) longer-term data suggest that biologically induced precipitation may be a means of actinide immobilization, but this needs to be investigated further.

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# PART 1: INTRODUCTION AND BACKGROUND

WIPP microbiology is unique among other deep geological repositories, in that it represents the intersection of hypersaline system ecology and repository ecology. The distinct differences in these two types of systems have presented challenges to investigations into the microbial influence on WIPP performance. Biocolloid transport is one such challenge. In order to support the use of finite biomass-based parameter values, background information on the biomass loading at the WIPP and the potential for these organisms to survive or influence the biocolloid parameter will be presented. The mechanisms of bioassociation will be described, and a literature review of WIPP-relevant association data will be presented.

## 1.1 Overview of WIPP Microbiology

### 1.1.1 What is there now?

Two populations of microorganisms exist within any repository: indigenous and introduced. At the WIPP, the indigenous microorganisms are those present in inter- and intra-granular fluids. Introduced organisms are those present in emplaced waste and those introduced on miners and mining equipment and via air intake shafts.

Different types of microorganisms have been cultivated from WIPP halite, depending on the concentration of salt used in the enrichment medium (Swanson et al., 2013a). At the higher salt concentrations, extremely halophilic archaea (class *Halobacteria*) were enriched; while, at lower salt concentrations, halophilic to halotolerant bacteria dominated. The haloarchaeal isolates can grow in saturated salt conditions, including WIPP brines, but only one bacterial isolate is able to do so (Swanson and Reed, 2018). Most of the bacteria isolated from WIPP halite grow at much lower salt concentrations (<1 M NaCl); although they have been shown to survive for weeks in WIPP brine without growth. These findings are consistent with those of others and reflect the known capabilities of each type of organism to cope with the osmotic stress of high salt (Oren, 2006, 2011; Franze and Cherkouk, 2016).

Introduced organisms have been less well studied. Investigations of the microbial population of a WIPP-bound waste drum yielded extremotolerant bacteria (phylum *Actinobacteria*) and spore-forming bacilli that did not grow at high salt concentrations, although they were able to survive for weeks (Swanson et al., 2015). It is possible that some of the bacteria isolated from WIPP halite (see paragraph above) were introduced from outside the repository, as the halite samples used for inocula were intentionally not surface-sterilized. Fungal spores and spore-forming bacilli are also present on drift wall surfaces and in the air and could have originated from outside the repository (Swanson et al., 2013a; Swanson and Reed, 2018).

### 1.1.2 What is expected over the lifetime of the repository

Which microorganisms will be present over the lifetime of the repository will depend upon which can survive and/or grow under the natural and man-made constraints of high ionic strength, anoxia, low water activity/high chaotropicity, alkaline pH, and possible radioactivity,

and which are capable of using the emplaced substrates and nutrients. The two main constraints will be high ionic strength and anoxia.

The microorganisms that inhabit subterranean salt deposits are unique in their ability to survive at high ionic strength by osmotically balancing their internal and external environments. Two strategies exist by which organisms can do this: 1) by importing high concentrations of $K^+$ and $Cl^-$ ions or 2) by accumulating low-molecular weight organics, called "compatible solutes." Extremely halophilic archaea (haloarchaea) and only two bacterial genera are capable of the first mode of osmoregulation. All other bacteria and eukaryotes employ the second mode. Both strategies require energetic input, but the second is much more costly than the first, especially if the solutes must be synthesized. As a result, the dominant microorganisms in hypersaline settings, such as the WIPP, are haloarchaea rather than bacteria.

The energetic cost of osmoregulation also constrains which modes of metabolism are favorable. As such, many anaerobic modes are limited or even effectively eliminated at high salt concentrations. There are no documented extremely halophilic, anaerobic organisms from subterranean salt deposits, including the WIPP (e.g., metal- or sulfate-reducing bacteria). While these organisms have been detected in surficial hypersaline settings, their activity is still constrained by increases in salt concentration (Kulp et al., 2007) and dependent upon interactions with naturally present halophilic organisms that provide them with other needed nutrients (e.g. algae, brine shrimp; Kelley et al., 2014).

Haloarchaeal longevity in subterranean halites (entrapped in fluid inclusions) is well documented; thus, these organisms are likely to be present throughout repository history even if they are not active (Mormile et al., 2003). However, few haloarchaea are capable of anaerobic growth, other than nitrate reduction, the reduction of methylated amines and sulfoxides (derived from compatible solutes), and the fermentation of certain amino acids (Oren and Trüper, 1990; Oren, 1999, 2011).

### 1.1.3 Predicted biomass concentrations

In addition to the above mentioned constraints on microbial activity and survival, the microbial populations at the WIPP will also be limited by the fact that the WIPP is a closed system. As in any closed system, microbial growth is limited by the depletion of substrates and nutrients or by the build-up of inhibitory by-products of metabolism; therefore, the biomass concentration cannot increase in perpetuity (Madigan et al., 2018; Pedersen, 2002).

The numbers of cells currently present within the WIPP is indeterminate. Several relevant matrices from the WIPP have been surveyed for microbial numbers. These are shown in Table 1.

| **Table 1. Cells Counts Measured from Various WIPP Matrices** | | | |
|---|---|---|---|
| Matrix | Francis and Gillow, 1993 | Vreeland et al., 1998 | Swanson et al., 2013a, and unpublished |
| Halite | Not done | Not done | $0 – 10^2$ cells/g (mostly 0 counts) |
| Brine | $7.2 \times 10^4$ cells/ml $3.4 \times 10^6$ cells/ml (open borehole G-seep; stain used for cell counting does not differentiate between live and dead cells) | Not done | 0 cells/ml (fresh brine seep; stain used for cell counting differentiates between live and dead cells) |

These counts are consistent with findings in other subterranean salt deposits (between 1 pg-10 µg biomass per kg of salt; Stan-Lotter and Fendrihan, 2011) and are generally less than counts obtained in surficial environments. The variation in halite cell counts is due to the heterogeneous distribution of cells within fluid inclusions or interstitial spaces (Norton et al., 1993; McGenity et al., 2000; Stan-Lotter and Fendrihan, 2011). The variation in brine counts may be a function of brine source and exposure to open air and mine workings. For example, no cells could be found in a fresh brine seep from WIPP (Swanson, unpublished); whereas, brine seeps from other boreholes contained many more (Francis and Gillow, 1993).

Cell numbers in actively growing cultures of WIPP-relevant samples are shown in Table 2. Numbers reported as “colony-forming units” (CFU) indicate that samples were plated onto solid growth media. Numbers reported as “cells/ml” indicate enumeration was performed by direct microscopy of aqueous samples.

**Table 2. Cells Numbers Measured in Actively Growing Cultures of WIPP-Relevant Samples**

| Matrix | Francis and Gillow, 1993, 1998; Gillow and Francis, 2006 | Vreeland et al., 1998 | Swanson et al., 2013a, 2013b, 2016, unpublished |
|---|---|---|---|
| Halite | Not done | 0- 6.9 x $10^3$ CFU/g halite, median 425 CFU/g (aerobic) | ~$10^5$-$10^9$ cells/ml in broth culture (aerobic); no growth (anaerobic) |
| Brine | 0.1 – 1.0 x $10^4$ CFU/ml G-seep, direct plating (aerobic) | 30 CFU/ml (DHP402A) to 1.0 x $10^4$ CFU/ml (G-seep), direct plating (aerobic) | 0 cells/ml (fresh seep from Salt Disposal Investigation area),in enrichment culture (aerobic) |
| Air | Not done | 0 CFU after driving along WIPP drift with exposed agar plate (aerobic) | 0 CFU on high salt plates after 24 hours of exposure to air in 3 locations with different air flow (aerobic); multiple colonies on low-salt plates, mostly fungal |
| Mixed matrix: muck pile salt, brine lake sediment and brine, halite | 5.12 x $10^5$ cells/ml (unamended, uninoculated) to 2.24 x $10^8$ cells/ml (amended, inoculated, excess nitrate; anaerobic) | Not done | Not done |

In none of the above cases have cell numbers exceeded $10^{10}$ cells/ml under optimum conditions for growth. The only inoculum matrix that yielded growth under anaerobic conditions contained surficial brine lake sediments (note: anaerobes have been enriched from WIPP area groundwater samples; Swanson et al., 2013a). Finally, cell counts under anaerobic conditions (Gillow and Francis, 2006) were an order of magnitude lower than those under optimal aerobic conditions (Swanson et al., 2013a).

To the authors' knowledge, there are no documented counts of cell numbers in radioactive waste, and so any predictions of biomass concentrations in waste would be highly speculative. Waste contents are varied, but the components most likely to contain microorganisms are soils. Unadulterated, healthy soils can contain anywhere between $10^8$-$10^{11}$ cells/gram (Torsvik and Øvreas, 2002; Raynaud and Nunan, 2014). In contrast, accounts of microorganisms in nuclear

waste contaminated environments report that numbers of cells were low—approximately $10^4$ CFU/g Hanford high level waste contaminated sediment (Fredrickson et al., 2004) and ~$10^5$ CFU/g of low-level transuranic (TRU) waste contaminated soil (Barnhart et al., 1980). This suggests that the soils in waste drums will contain far fewer cells than a healthy soil. Other nuclear waste contents include dried organic sludges, but as with the contaminated soils, the numbers of cells therein would be limited by desiccation and radioactivity (Barnhart et al., 1980; Zavilgelsky et al., 1998; Fredrickson et al., 2004; Swanson et al., 2015).

The authors are unaware of any data on the numbers of organisms that may have been, and are still being, introduced through mining operations or through air intake. In other deep geological repositories (DGR), this distinction between indigenous and introduced can be difficult, since they cannot use halotolerance as an inclusion criterion; thus, in any DGR settings it is preferable to address the long-term viability of any organism that is present. However, even at the low ionic strengths in those repository settings, it was shown that introduced bacteria did not establish themselves under the in situ DGR conditions (Pedersen et al., 1999).

The importance of dead cells in deep geological repositories has not been an area of focus for repository microbiology research. At most, it is addressed when considering actinide retention in biofilms (Pedersen, 2002). As a result, the authors have not found any published data on the quantity of dead biomass at the WIPP and only one mention of the lack of dead biomass in Yucca Mountain (Wang and Francis, 2005).

## 1.2 Bioassociation

### 1.2.1 Mechanisms of association and biological influence

In this report, the term “bioassociation” will be used as an umbrella to refer to all types of microbial-actinide associations, whether internal or external and whether transient or accumulative. If the specific mode of interaction is known, it will be named. Ultimately, the nature of the association is important in determining the long-term fate of the actinide, but this determination may not always be feasible, given the lifetime of the WIPP.

Surface sorption is a metabolism-independent process in which metals interact with functional groups at a cell’s surface, either by ion-exchange or electrostatic interactions. This process can be reversible depending on the pH and the presence of strong complexing agents. It is generally pH-dependent, as functional groups become deprotonated with increasing pH. At normal physiological pH, cells generally carry a net negative surface charge. Surface sorption can occur on live and dead cells, on live but inactive cells, and onto cell debris (e.g., extracellular polymeric substances, or EPS). It can lead to either the mobilization or immobilization of an actinide, depending on the state and mobility of the biomass

Biomineralization refers to the induction of mineral formation by microorganisms and can occur both internally and externally to the cell. Generally, it involves the association of metal with phosphate or carboxylate groups. Once the metal has bound, it can serve as a nucleation site that furthers the progression of mineralization. Often, the release of phosphate is a toxic response by the organism to sequester the metal. Phosphate mineralization is usually induced by active cells,

as the phosphatase enzyme is required, but cells do not necessarily need to be growing for this to occur. In general, mineralization leads to the immobilization of an actinide, as cells or the actinide solid phase precipitate from the matrix.

Internal uptake is the introduction of actinides into the cell. Most cells will respond to metal exposure with an active efflux system. This means of detoxification is less costly than sequestration that may require induction of a complexant, such as phosphate. Still, internalization as a sequestered metal is possible and can lead to high concentrations of the metal inside the cell (i.e., bioaccumulation). If the actinide is bound to a ligand that is biodegradable, its fate is uncertain. It could be taken up into the cell but could also be ejected again. Thus, uptake can be either transient or accumulative. Finally, there are some radionuclides (Cs and Sr) that can substitute for biologically important cations (K and Ca, respectively) without inducing a toxic effect. Internal uptake can lead to either mobilization or immobilization, depending on whether the organism itself is mobile.

### 1.2.2 WIPP-relevant literature review

To date, there are still relatively few publications on the bioassociation of metals or actinides that are relevant to all the unique conditions at the WIPP—e.g., high ionic strength, alkaline pH, presence of waste complexants, use of halophilic organisms, etc. The following section is a summary of studies using relevant organisms at relevant ionic strength. Conditions of pH in most of these studies are rarely comparable to WIPP conditions but were used to simplify and better control actinide chemistry and speciation.

#### 1.2.2.1 An(III) and analogs

Francis et al. (1998) reported a significant difference in Am-EDTA (ethylenediamine tetraacetate) association with the halophilic bacterial isolate from the WIPP environs, *Halomonas* sp. WIPP-1A, and a mixed culture of extreme halophiles, BAB (brine A bacteria), during growth experiments (pH 6.3). The bacterial culture removed ~95% of the added Am from solution; whereas, the presumably haloarchaeal culture removed only 28%. The authors did not ascribe specific reasons for these differences but attributed the overall variation to differences in the organisms themselves and to variations in actinide speciation in the growth medium and in the presence of complexants.

Takenaka et al. (2004) measured increasing log $K_d$ values for europium and curium onto the moderately halophilic bacterium, *Halomonas elongata*, as the NaCl concentration increased (pH 5). They attributed this finding to an increase in cell surface hydrophilicity and changes in morphology, leading to an increase in specific cell surface area, as ionic strength increased.

Ozaki et al. (2004) examined the sorption of europium and curium onto *Halomonas* sp. WIPP-1A and two strains of the haloarchaeon, *Halobacterium salinarum*. Although conducted at high salt concentrations (3.4 M NaCl and 4.3 M NaCl for the bacterium and archaea, respectively), the experiments were at low pH (3-5) in order to avoid hydrolysis. The authors measured fast association kinetics (within 10 minutes for the haloarchaea and 20 minutes for the bacterium) that increased with increasing pH up to 75% of the added element. The authors also reported an

increase in sorption affinity with the excretion of EPS; although, the presence of EPS was not verified.

The interaction of *Halobacterium noricense* Deutsche Sammlung von Mikroorganismen (DSM) strain 15987 with curium and europium has been investigated and determined to be more than simple surface complexation (Bader et al., 2019). After 24 hours, europium was found in both aqueous and solid phosphate complexes. The lower concentration of Eu tested resulted in higher association (~70%); while, the higher concentration of Eu had a toxic effect that led to cell clumping. Still, up to 30% of the added Eu was associated with the cell aggregates and was shown to be complexed with phosphate groups, presumed to be in EPS. Curium was associated with both phosphate and carboxyl groups, and the resulting species changed with both time and $pC_{H+}$.

The removal of +3 actinides via enzymatically derived phosphate has been shown repeatedly in experiments at lower ionic strengths (Macaskie et al., 1994). This mechanism of detoxification is apparently also present in extreme halophiles, including archaea. Phosphate complexes with actinides and metals can also occur via the phosphate component of nucleic acids or phospholipids present in EPS, including at high ionic strength (Hufton et al., 2016; Vreeland et al., 1984).

The association of the +3 analog, neodymium, with *Chromohalobacter* sp. increased as the $pC_{H+}$ increased, over a narrow range, in both the presence and absence of EDTA (Reed et al., 2013). Association of Nd with the organism was lowered by approximately 25% in the presence of EDTA.

Although not halophilic or even halotolerant when vegetative, spores of a *Bacillus* sp. isolated from WIPP-bound waste were tested for their ability to adsorb neodymium under low and high salt conditions (Hazelton and Swanson, 2017). Association decreased with increasing ionic strength, and in both low and high salt conditions, the amount of Nd associated decreased over time. This was hypothesized to be due to the release of a complexing ligand, dipicolinic acid (DPA), by the spores in the presence of high concentrations of chloride. This same inverse correlation with ionic strength has been shown previously for spores of another *Bacillus* sp. (Gorman-Lewis et al., 2013). This study also showed that DPA can be involved in neptunium mobility.

#### 1.2.2.2 An(IV) and analogs

. Francis et al. (1998) found that ~74% of the Th-EDTA present in the fluid column was associated with WIPP-1A, but none associated with the BAB culture (both at pH 6.3). There was also a toxic effect observed for the bacteria but not the archaea. They dismissed most of their Th-nitrate data due to precipitation.

Thorium interaction with the WIPP halophilic isolates, *Chromohalobacter* and *Halobacterium* sp., was found to be low for both and mitigated somewhat by the presence of EDTA (Reed et al., 2013). Association was also $pC_{H+}$ dependent, with the lower pH test conditions found to be less susceptible to precipitation effects. In the presence of EDTA, *Chromohalobacter* associated with

33% and 57% of Th at $pC_{H+}$ 8.6 and 9.3, respectively. In the absence of EDTA, association increased to 54% and 81%, at those same $pC_{H+}$ conditions.

Others have studied Pu(IV) at lower ionic strengths and/or acidic pHs to simplify the Pu(IV) chemistry. At pH 2.7-2.9 and low ionic strength (0.08M NaCl), Druteikienė et al. (2010) showed that organisms isolated from a low-level radioactive waste site were capable of sorbing significant quantities of Pu(IV)—35% for a *Bacillus* species and 70% for a *Micrococcus* sp. However, these conditions are far from WIPP-relevant.

#### 1.2.2.3 An(V)

Studies of An(V) bioassociation at high ionic strength are also scarce. Francis et al. (1998) measured low association of Pu(V)-EDTA with WIPP-1A and BAB (9% and 2.5%, respectively; pH 6.3). In contrast, Np(V)-EDTA associated more with BAB (37%) than with WIPP-1A (12%).

Ams et al. (2013) studied the interaction of Np(V) with *Chromohalobacter* sp. over a broad pH range and at two ionic strengths (2 and 4 M perchlorate). Association at lower pH was controlled by cell surface interactions with the neptunyl ion; whereas, at high pH, negatively charged Np-carbonate species led to a decrease in association. Both the low and high ionic strength tests resulted in a maximum at roughly pH 7.5. Association was greater at the higher ionic strength (89% compared to 63%) and reported to be due to greater ion activity.

#### 1.2.2.4 An(VI)

Uranium is the most commonly studied radionuclide in biosorption investigations at both low and high ionic strength. Gillow et al (1999, 2000) investigated the association of complexed uranium with WIPP 1A. They reported that 32% of added U-nitrate most likely interacted with carboxylate groups at the cells' surfaces and was also internalized in granules, but only at pH 5. Uranium-EDTA precipitated at all the tested pHs (5, 7, and 9), as did U-nitrate at pH 7 and 9. Neither U-citrate nor U-carbonate were taken up at any pH tested (5, 7, 9 for citrate; 9 for carbonate).

Francis et al. (2004) studied three halophilic organisms: *Halomonas* sp. WIPP-1A, *Halobacterium salinarum* (then *halobium*), and *Halanaerobium praevalens*. *Halobacterium* associated with 90% of the added uranium, *Halanaerobium* with 73%, and *Halomonas* with 58%, which coincidentally also corresponds to the decrease in ionic strength of the test matrices. In all cases, uranium was shown by transmission electron microscopy with energy dispersive X-ray spectrometry (TEM-EDS) to be localized at the cells' surfaces. In addition, for *Halomonas*, the uranium was localized in phosphate granules within the cells. The cell lysate of *Halomonas* was also tested for its ability to sorb uranium; it associated with 58% of the added uranium, which was shown by TEM-EDS to be with carboxyl groups at the walls and within ejected phosphate granules.

Bader et al. (2017, 2018) showed that uranium complexes with surface carboxyl and phosphoryl groups on two *Halobacterium* spp.: *Halobacterium noricense* DSM 15987 and the WIPP strain.

When testing the DSM strain, they found concentration-dependent and time-dependent speciation and mineralization of uranium: phosphate minerals at low U concentration and carboxyl interaction at high U concentration. The formation of cell aggregates was theorized to be a stress response, leading to the sequestration of added uranium.

#### 1.2.2.5 Other

The association of non-radioactive metals with carboxyl groups has been investigated using other halophiles. Kenward et al. (2013) observed the precipitation of dolomite on both intact cells and cell walls of *Haloferax sulfurifontis* over a period of several weeks. This was attributed to a high density of carboxyl sites leading to the dehydration of Mg ions, such that an initial nucleation site could be formed. Showalter et al. (2016) used the WIPP strain of *Halobacterium* to test the sorption of Cd and hypothesized its association with thiol groups at the cells' surfaces. Popescu and Dumitru (2009) found that EPS produced by strains of *Haloferax* were capable of binding heavy metals (e.g., Pb, Cr), proposing their use for bioremediation.

The ability of dead biomass to adsorb heavy metals is well established. However, with the exception of Gillow et al. (1999) and Francis et al. (2004), there are no studies that have measured the ability of dead (i.e., lysed) cells to adsorb actinides under remotely relevant WIPP conditions. However, Bader et al. (2017) suggested that the dead cells enmeshed in the aggregates precipitated from suspension and could be considered immobile.

## PART 2. WIPP CCA BIOCOLLOID MODEL

Biologically enhanced transport of radionuclides away from a repository setting is one of several processes that could affect repository performance. This section will review the WIPP biocolloid model and its assumptions, experiments that led to the derivation of parameters for the original certification and subsequent recertifications, and the uncertainties that prompted the introduction of a biomass-based approach for CRA 2014 (US DOE, 2014). A comparison of the CCA and CRA 2014 approaches is presented.

### 2.1 Biocolloid Model

The WIPP Performance Assessment (PA) considers the enhanced transport of actinides by microorganisms (i.e., biocolloid transport) to be a significant, yet uncertain, contribution to the total mobile actinide concentration. It is one of four colloidal contributions to the source term and is calculated as a proportion of the dissolved concentration of each actinide that is associated with biomass (DISSOLVED * the proportionality constant, PROPMIC) and is bound by an upper limit (CAPMIC) that caps the microbial colloid contribution.

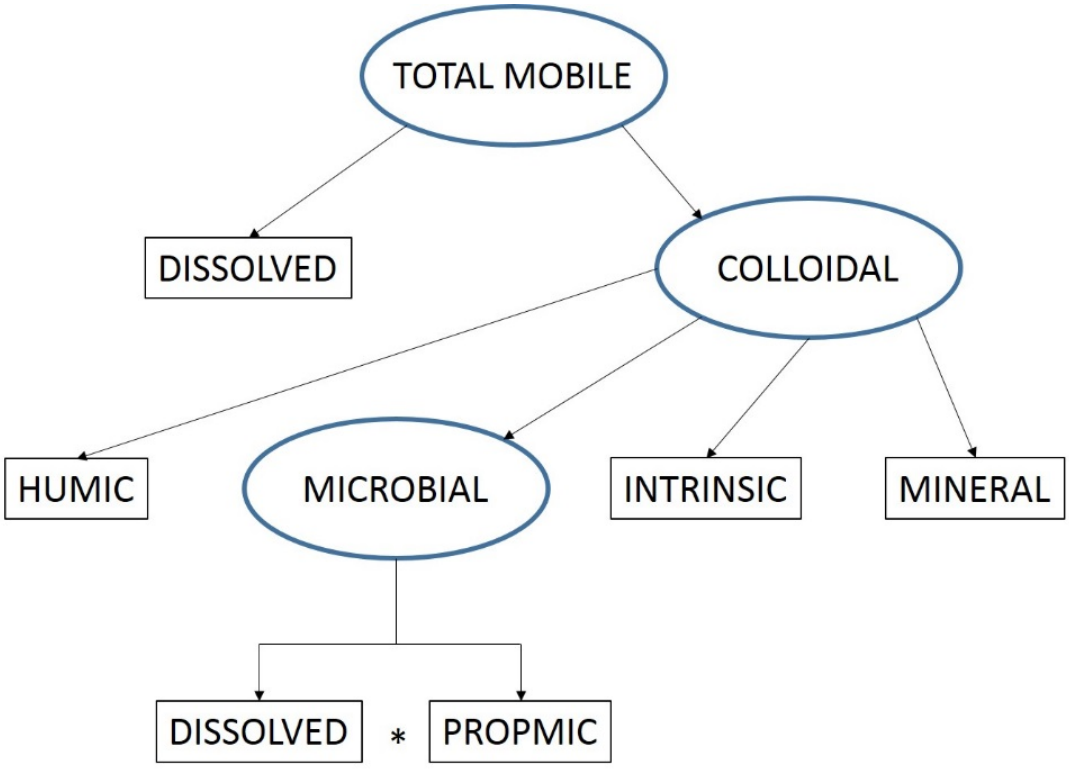



Figure 1. Schematic of the PA colloidal model.

### 2.1.1 Assumptions

There are two key assumptions that have been made in PA regarding the biocolloid contribution to the source term: 1) that all microbes associate with actinides; and 2) that all microbes are mobile. In addition, PA treats each actinide's bioassociation potential separately. These assumptions and treatment allow a safe, although less than realistic, overestimation of the microbial contribution.

First, bioassociation behavior can differ between organisms of different types, organisms in different stages of growth, at different pH, in the presence of ligands or competing cations. Some have shown that even among a population that can adsorb actinides, only a certain percentage might actually do so (Strandberg et al., 1981).

Second, not all cells are mobile. Cells can exist in a planktonic state or attached to surfaces, such as in the form of a biofilm. Indeed, it is usually to the organism's advantage to adhere to a surface for many reasons, such as easier access to nutrients or protection from predation or adverse environmental conditions (Madigan et al., 2018). Many organisms also grow in clumps leading to sedimentation. Certain types of biological influence, such as biomineralization at the cell's surface, are presumed by many repository microbiologists to also lead to immobilization of metals via precipitation or biofilm incorporation (Pedersen, 2005).

### 2.1.2 Conceptual model definitions for PROPMIC and CAPMIC parameters

Conceptually, the microbial contribution to the mobile actinide source term is the concentration of actinides that is associated with the microorganisms suspended in brine, and hence deemed mobile, under a direct brine release scenario (US DOE, 1996; Papenguth, 1996; Francis et al., 1998; US DOE, 2009; US DOE, 2014).

The specific definitions in the original WIPP biocolloid model for the PROPMIC and CAPMIC parameters are (Papenguth, 1996):

> PROPMIC: the proportionality constant that describes "the amount of actinide element bound to mobile microbes" and is calculated as "the ratio between the microbial actinide and dissolved actinide"
>
> CAPMIC: "the maximum concentration of actinide that can be associated with mobile microbes"

In practice, these parameters might be derived using different measurement approaches, but the definitions for the parameters in the conceptual model remain the same, regardless of the means of measurement. PROPMIC has routinely been calculated as "the ratio between the microbial actinide and dissolved actinide", as measured by filtration, in both past and current work. For CCA through CRA 2009, CAPMIC was derived from toxicity studies and measured as the actinide concentration "at which no growth was observed." In the current CRA, the CAPMIC values are derived from the concentration of actinide associated with a fixed biomass concentration, such that the number of microbes imposes the limit on the microbe-bound actinide concentration.

## 2.2 Experiments in support of CCA

### 2.2.1 Biosorption

In order to generate values for the microbial term in Figure 1, a set of experiments was conducted by Francis and colleagues (Papenguth, 1996; Francis et al., 1998). The WIPP-1A bacterium was inoculated into growth media containing complexed actinides (pH 6.3) and incubated until stationary phase. Samples were then withdrawn from the fluid column and fractionated by filtration. The 0.03 µm filtrate represented the total dissolved actinide concentration; while, the fraction between 0.4 -10 µm represented the mobile, microbially-associated actinide concentration. Similar experiments were carried out with the BAB culture but were not used to determine parameter values, as these cultures required a longer period of time for growth.

### 2.2.2 Toxicity

In addition to sorption experiments, Francis et al. conducted growth inhibition studies, in which the same cultures were exposed to different concentrations of complexed actinides in the same growth medium as the sorption experiments. The extent of growth was measured with a final optical density reading and, where feasible, a final direct microscopic cell count. Cell counts were not obtained for Th, Np, or Am, and optical density (OD) readings for higher concentrations of Th and U were affected by precipitates.

### 2.2.3 Derivation of parameter values

The proportionality constant, PROPMIC, was generated by dividing the moles of microbially-associated actinide (0.4 µm filter retentate) by the moles of dissolved actinide (0.03 µm filtrate) in the sorption studies. This value was then multiplied by the expected dissolved concentration of actinide in brine to determine the microbial contribution to the total mobile actinide concentration.

The actinide concentration at which growth was no longer observed was used as a measure of the CAPMIC value, which was defined as the maximum concentration of actinide that can be associated with mobile (proxy for “viable”) microbes. In cases where complete inhibition was not observed, CAPMIC values were reportedly determined by linear extrapolation and the addition of an order of magnitude for uncertainty (Papenguth, 1996). This could not be verified for the plutonium data.

## PART 3. 2014 CRA BIOCOLLOID MODEL

### 3.1 Experiments in support of CRA 2014

A series of experiments was conducted to provide additional data for the 2014 CRA biocolloid parameters. These experiments focused on a haloarchaeal isolate (*Halobacterium* sp.) deemed to be more relevant to the repository environment than most bacteria but also focused on the moderate-to-extremely halophilic bacterium, *Chromohalobacter* sp. The experiments were designed to measure sorption phenomena and did not focus on other forms of bioassociation. WIPP brines and formulations specific to a range of $pC_{H+}$ values were used as the test matrices. From these experiments, new parameters for PROPMIC and CAPMIC were derived and used for the CRA 2014 PA.

### 3.2 Justification for the parameter changes

The parameter changes in CRA 2014 were made for the following reasons:

- To better reflect the understanding of the microbial ecology at the WIPP and, more specifically, limits to survival or activity under WIPP-relevant conditions
- To improve the consistency of the approach with the understanding of actinide chemistry and speciation under WIPP-relevant conditions

A comparison of the two approaches for deriving parameter values is provided in Table 3.

**Table 3. Comparison of Experiments for Deriving Parameter Values for the Original CCA and CRA 2014**

| EXPERIMENTAL CONDITION | PAST EXPERIMENTS FOR CCA | EXPERIMENTS FOR CRA 2014 |
|---|---|---|
| State of cells (phase) | Experiments used growing cells; samples taken at late log/early- and mid-stationary phase | Experiments used washed cells in stationary phase ("resting") |
| Length of experiment | Data used were from stationary phase (7-15 days) | 2 hours to 2 weeks |
| Actinide complex | Actinides added were complexed with ligands (EDTA, nitrate, citrate (phosphate was present in growth medium) | Effects of EDTA were studied, but most experiments did not use ligands |
| Actinide species | Unclear speciation with pH and redox changes | Redox-invariant analogs with defined pH-specific speciation |
| Test matrix | Semi-defined medium (i.e. some components, such as yeast extract, were "complex" and could possibly contain ligands | Simplified brines and $pC_{H+}$-specific brines |
| Means of cell separation | 0.4 micron filtration | 100 kD filtration |
| Comparison to WIPP conditions | WIPP-relevant conditions unclear at the time of this work (i.e., ligand use not based on inventory, MgO buffering not taken into account); pH ~6-8 | Using WIPP-relevant brines at relevant $pC_{H+}$, EDTA based on inventory |

Since those changes were introduced, questions have arisen regarding the validity of a biomass-based CAPMIC value. Because CAPMIC is a measure of the maximum concentration of microbially-mobilized actinide, it should be a function of the maximum concentration of biomass available to associate with the actinide and the maximum concentration of actinide that is bioavailable. The maximum available biomass, as discussed in Part 1, is dictated by repository conditions and cannot increase in perpetuity. The latter, actinide bioavailability, is limited by inventory, solubility, and the presence of ligands and other competing metals (Van Soest, 2015, 2018; Brush and Domski, 2013).

In theory, bioassociation increases with increasing biomass to the point where all the actinide has associated and increasing the concentration of the actinide will increase the amount associated until all sites are saturated (for surface complexation), or until organism activity is detrimentally affected and it can no longer take up the actinide or generate phosphatase (internalization,

enzymatically-driven mineralization). In either case, association is limited by the "bio" component present.

### 3.3 Uncertainties in measurements for the biocolloid parameters

Both the toxicity-based and the biomass-based approaches have associated uncertainties. These arise from methodology, variability in organism responses, and the use of different test matrices. The following section highlights the chief sources of uncertainty for both approaches and provides a summary comparison.

#### 3.3.1 Using optical density (OD) as a proxy for cell numbers

This is usually an acceptable method to measure biomass (for the linear part of the growth curve), except in cases where precipitation occurs. In previous work, for example in the Th and U experiments for CCA (Papenguth, 1996), some values were rejected based on precipitation interfering with OD readings; yet, one of those values were chosen as the Th CAPMIC, since no cell counts had been obtained. In WIPP brines, microbially-induced precipitation can also occur, leading to false readings. In these cases, direct microscopic cell counts are the preferred method for measuring biomass and can also take into account some dead or dying cells.

#### 3.3.2 Variations between different types of organisms

Some organisms will be more resistant to actinides than others, even organisms that are representative of the WIPP environs. For example, the growth of WIPP-1A was much more susceptible to uranium and neptunium than was the BAB culture; whereas, BAB growth was more susceptible to americium and plutonium than WIPP-1A at the concentrations tested (Francis et al., 1998). Parameters for CCA were derived from WIPP-1A results.

Bioassociation behavior can also vary between organisms (Francis et al., 1998; Bader et al., 2018). Francis et al. measured different levels of association between the archaeal culture than the bacterial; while Bader et al. determined that different functional groups were involved in uranium association with a haloarchaeon and a bacterium isolated from halite. Some of these differences may reflect different cell wall structures. Most archaea (and some bacteria) possess a crystalline, proteinaceous S-layer that is responsible for much of the measured association. Many, but not all, organisms can secrete EPS which can also take up actinides. Additionally, the loss of cell wall integrity at higher ionic strengths can present as apparent sorption but may actually be internal uptake.

#### 3.3.3. Variations based on actinide speciation

Actinide speciation influences both toxicity and bioassociation, due to differences in chemical behavior. However, the test conditions often required to control for speciation are not always ideal for the organisms' growth or survival (e.g., acidic pH). This makes it difficult to extrapolate the results of experiments performed under ideal organism conditions to actual repository-relevant conditions, and vice-versa.

#### 3.3.4 Variations with complexation or between complexants

Some complexants will be used as substrate by many organisms (e.g., citrate) and will be taken up into cells, leading to a different effect when compared to those organisms that do not use the

ligand as substrate. The lack of a complexant could also lead to higher toxicity due to the presence of free metal ions. Studies with EDTA as the complexant have clearly shown that its presence reduces the amount of actinide associated with the organism (Reed et al., 2013). The reduction in bioavailability of an EDTA complex also leads to lower observed toxicity.

Additionally, the presence of EDTA can lead to the shedding of archaeal S-layers and changes in cellular morphology. Both of these effects can result in changes to measured bioassociation.

### 3.3.5. Variations with test matrix

The presence of other cations in the test matrix (specifically Mg) can affect actinide availability and toxicity, as they can compete for available ligands, thereby changing actinide speciation in solution. Ionic strength has also been shown to affect the extent of bioassociation, due to differences in actinide ion activity or cellular responses to different salinities (Ams et al., 2013).

### 3.3.6 Uncertainty in the state and numbers of cells at the WIPP

No one knows what the true numbers or condition of the microorganisms present in the WIPP will be at closure, at drum breach, or at inundation. Whether growing or resting, healthy or stressed, in high numbers or low numbers, cells will eventually be exposed to actinides. Toxicity may not guarantee a total loss of biomass, if the starting inoculum is high enough or healthy enough. Exposure during a resting state may not induce the same level of toxicity as exposure during growth.

To illustrate this uncertainty for toxicity, the cell numbers in the original CCA experiments never fell below $10^5$ cells/mL, even when toxicity was deemed “extreme”, and never fell below the original inoculum concentration within the time frames tested (Strietelmeier et al., 1999).

### 3.3.7 Summary of uncertainties

A comparison of both approaches, based on the above uncertainties, is provided in Table 4. This table highlights that the biomass-based approach is susceptible to less variability than a toxicity-based approach.

**Table 4. Comparison of Uncertainties for both Approaches to CAPMIC Parameters**

| SOURCE OF UNCERTAINTY | CCA EXPERIMENTS: SUSCEPTIBLE, Y/N? | CRA 2014 EXPERIMENTS: SUSCEPTIBLE, Y/N? |
|---|---|---|
| Methodology: use of OD | Y | N |
| Organism variability | Y (used bacterium) | Y (used archaeon) |
| An speciation | Y | N |
| Complexants | Y | N |
| Matrix | N (but not WIPP-relevant) | Y |
| Condition of cells | Y (relied on growth) | N (fixed and resting) |

## PART 4. RESULTS OF RECENT EXPERIMENTS: +3 CASE

Investigations into association behavior of the actinides with haloarchaea have continued. These experiments set out to address the issue of the long-term disposition of the actinide and to strengthen the argument for a biomass-based approach.

### 4.1 Experimental design

Recent work has focused on the +3 analog, neodymium, and its interaction with the WIPP isolate, *Halobacterium* sp. Most of this work was conducted in 3.42 M NaCl simplified brine at pH ~6, in order to establish behavior under controlled conditions. Later this was extended to simulated WIPP brines at 90% formulation. The experimental design of this work was similar to previous studies in this lab, with the following modifications:

- Biomass concentrations were typically measured in three ways: 1) as cells/ml via direct microscopic counts with Live/Dead staining; 2) as cell wet weights; 3) as optical density values at 660 nm. The latter measurement was used to target a specific biomass but was not used to calculate the final concentration.
- Experiments were run for extended time frames, up to 1-2 months

Cells were harvested at stationary phase, washed three times with 3.42 M NaCl solution at the target pH, and stored overnight at 4°C. Pellets were resuspended the following day to achieve the target OD and were then combined 1:1 with a test brine-neodymium solution containing twice the desired neodymium concentration. Typically the target concentration was ~12 µM, but in experiments with WIPP brines, the target was much lower (0.2 µM), due to solubility issues.

Cell suspensions were placed on a rotator, and samples were withdrawn periodically for analysis by ICP-MS. Samples were passed through 100kD centrifugal filters prior to dilution in 2% nitric acid. Measurements of Nd in the filtrate are considered to be “dissolved” Nd. As with previous work, the filtration step can capture surface sorption, internal uptake, and induced precipitation and mineralization.

### 4.2 Summary of results

Five experiments were conducted to test different aspects of bioassociation: kinetics, biomass-dependence, neodymium concentration dependence (all in simple brine); kinetics in WIPP-specific brines, and association behavior in the presence of EDTA, also in WIPP-specific brines. All data are presented as the loss of neodymium in solution, in both biotic and abiotic samples, and as the percentage of neodymium associated with biomass, relative to the abiotic control. In contrast to earlier work, results from these experiments showed slow "association" kinetics (up to and greater than 24 hours) that suggest another mechanism is involved besides, or in addition to, surface complexation.

**Kinetics.** In a simple kinetics experiment, two biomass concentrations were used for scoping purposes ("low," ~$10^8$ cells/ml; "high," ~$10^9$ cells/ml). After one month of exposure, the low biomass samples resulted in 53% association (Figure 3). In contrast, the high biomass samples resulted in ~60% loss of Nd from solution almost immediately, followed by a progressive and complete loss over 24 hours (Figures 2 and 3).

It is possible that the initial (see t = 0), rapid loss reflects surface complexation, but the mechanism for continued loss from solution is still under investigation. As a result, the term "biological influence" on Nd in solution is preferred to "association." This term can also include induced precipitation. These slow kinetics are in agreement with the findings of others with this same organism (Showalter et al., 2016; Bader et al., 2017) but are in contrast to those of WIPP isolate, *Chromohalobacte*r sp., that show a rapid association requiring 3 hours or less.

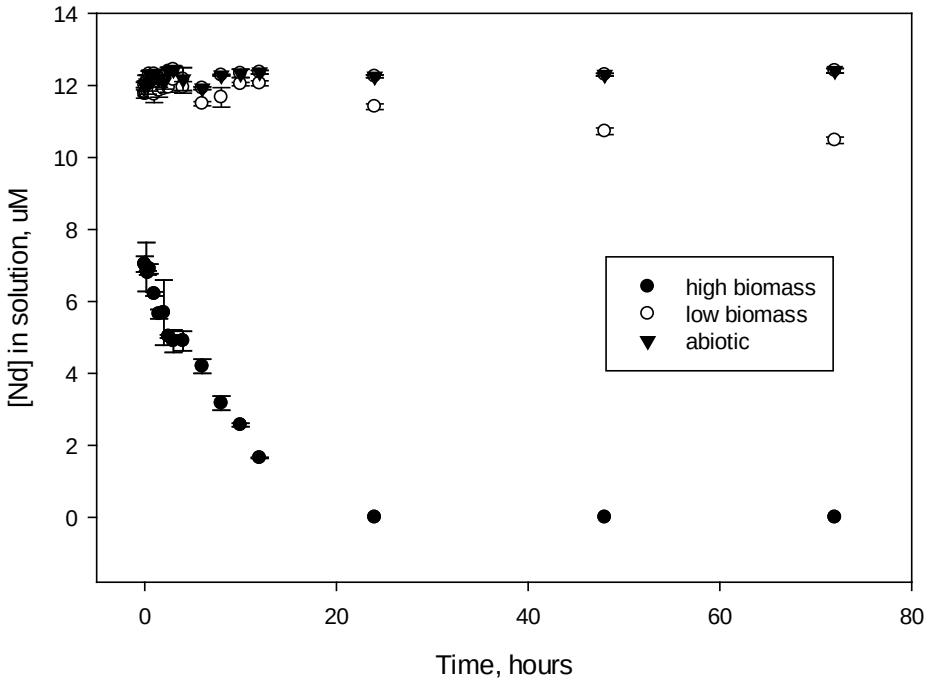



Figure 2. Loss of neodymium from solution containing two biomass concentrations, high (5.03 ± 0.88 x $10^9$ cells/ml) and low (2.02 ± 0.41 x $10^8$ cells/ml), compared to the abiotic control.

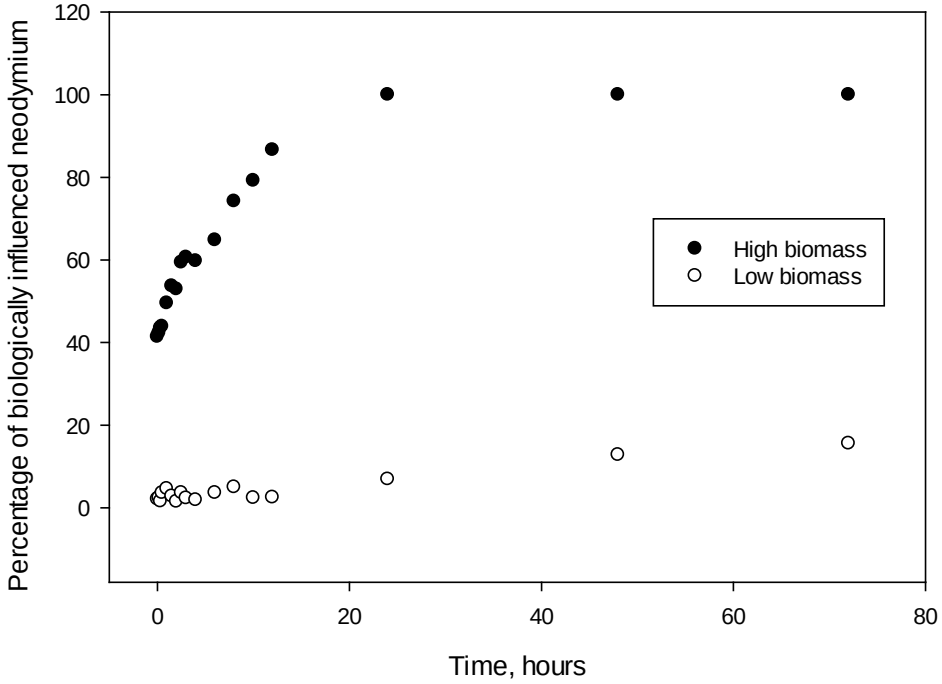



Figure 3. Percentage of biologically influenced neodymium in high and low biomass samples.

**Biomass dependence**. A biomass dependence experiment was also conducted with *Halobacterium* sp. and neodymium (Figures 4 and 5). In this experiment, biomass concentrations were varied by serial ten-fold dilutions, but the concentration of Nd was kept constant. Higher apparent association occurred at higher biomass concentrations, but the difference between higher and lower biomass association narrowed over time for the highest biomass loads tested ($10^{10}$ and $10^{9}$ cells/ml). At the concentration recommended for CAPMIC derivation ($10^{9}$ cells/ml), apparent association reached 100% at one month. At $10^{7}$ cells/ml, association was measured at only 6% and remained low over time; while at the biomass concentration similar to that measured in G-seep brine by Francis and Gillow ($10^{6}$ cells/ml; 1993), there was no biological influence on the neodymium in solution.

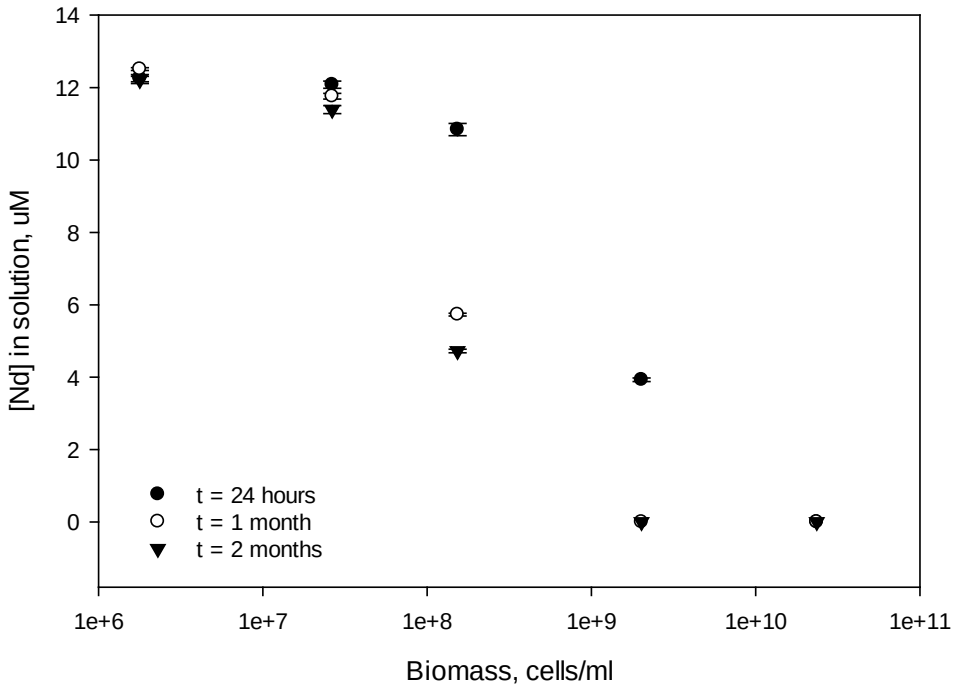



Figure 4. Neodymium in solution as a function of biomass concentration at three exposure times.

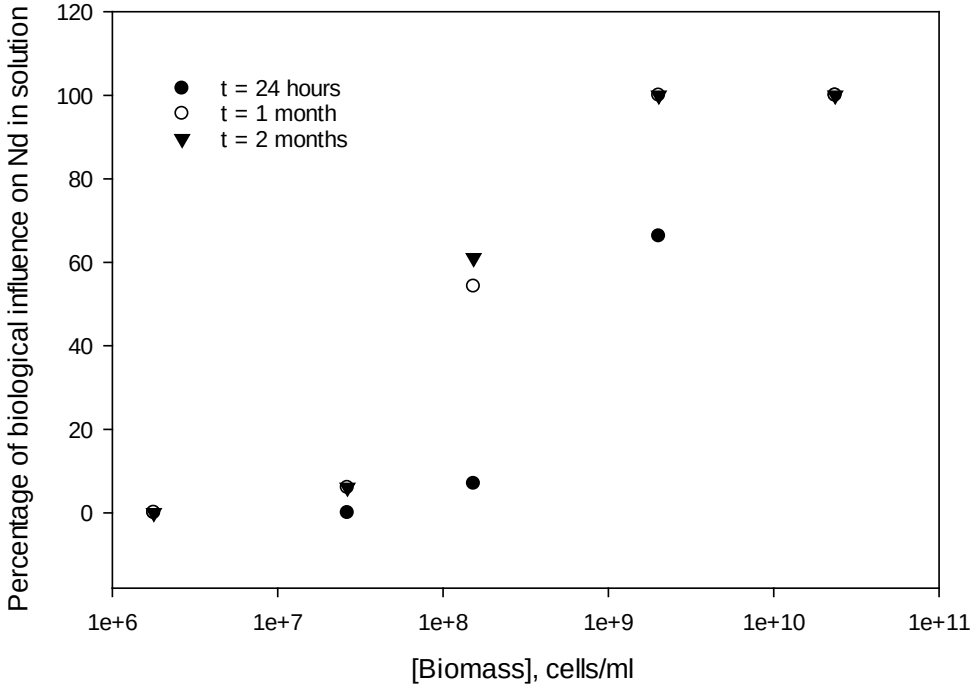



Figure 5. Percentage of biologically influenced neodymium as a function of biomass concentration and over time.

**Neodymium concentration dependence**. In experiments testing the influence of Nd concentration on association, the amount of Nd added was varied, and the biomass concentration was kept constant. In these experiments, lower starting concentrations resulted in higher initial association. However, by one month, all concentrations of Nd tested were completely removed from solution (Figures 6 and 7).

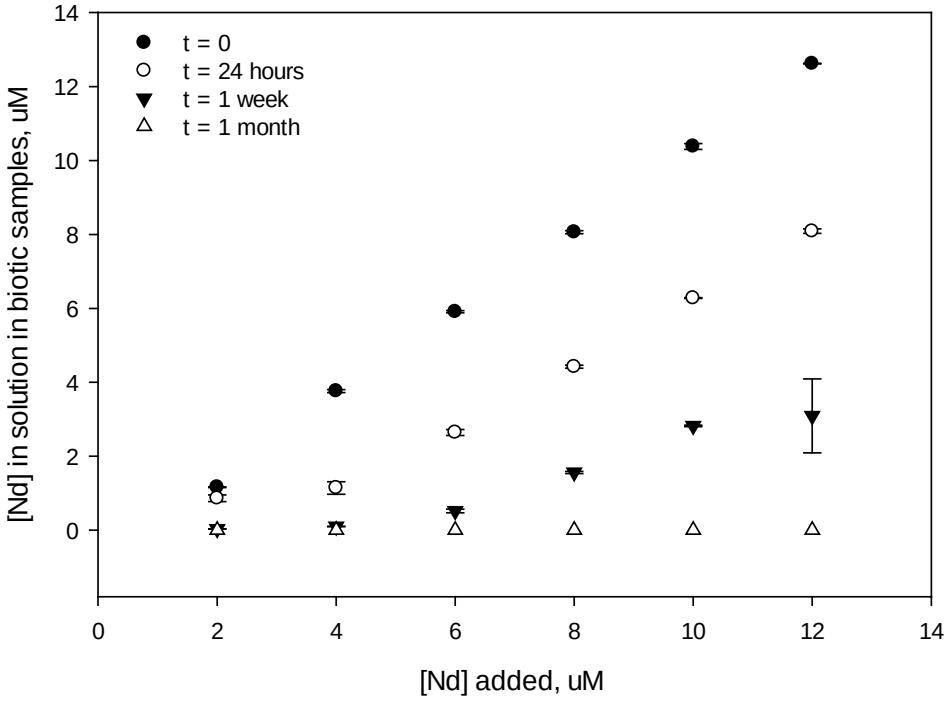



Figure 6. Neodymium in solution as a function of neodymium originally added to the biotic samples ($1.50 \pm 0.75 \times 10^9$ cells/ml).

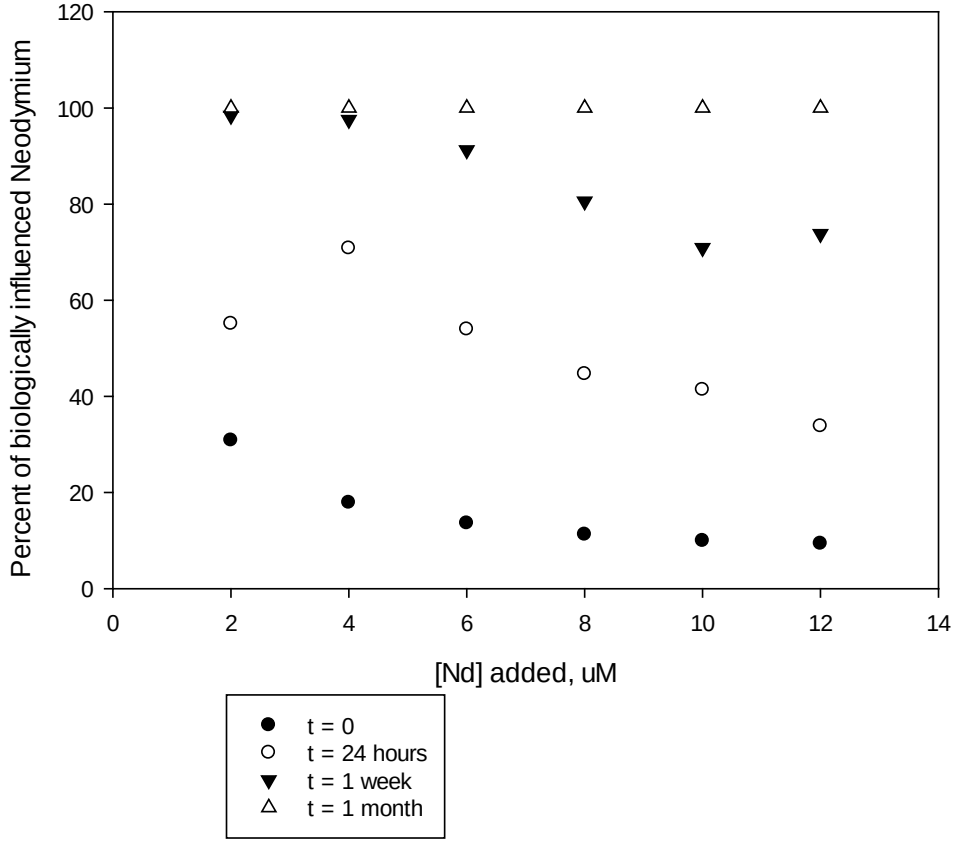



Figure 7. The percentage of added neodymium influenced by biomass as a function of Nd added.

**Kinetics in WIPP brines (No EDTA)**. The previous experiments were conducted in simple NaCl brine at the optimum concentration for *Halobacterium* sp. (3.42 M). Extending these experiments to WIPP-specific brines (90% formulation, anoxically prepared) with different compositions and pH yielded significant differences. Little to no association was measured in GWB and $pC_{H+}$ 9.5-specific brines; whereas, Nd was gradually lost from ERDA over an extended time (Figures 8 and 9). At the $pC_{H+}$ values of the brines (GWB 8.4; ERDA 8.8) and in the absence of carbonate, Nd is expected to exist as the hydrolysis species, $NdOH^{2+}$, and to a lesser extent $Nd(OH)_2^+$ and $Nd(OH)_3$ (aq) (Borkowski et al., 2009; Neck et al., 2009). Thus, neodymium speciation is unlikely to be the reason for the differences observed in this experiment. Instead, it is hypothesized that the differences in association are due to brine chemistry, specifically the presence of higher concentrations of magnesium in GWB and $pC_{H+}$ 9.5 brine possibly acting as a competing cation for cell surface sites but also affecting cell surface structures.

Qualitative changes in cell morphology and integrity were observed microscopically over time in ERDA but not in the other two brines. The final disposition of Nd under these conditions is still under investigation.

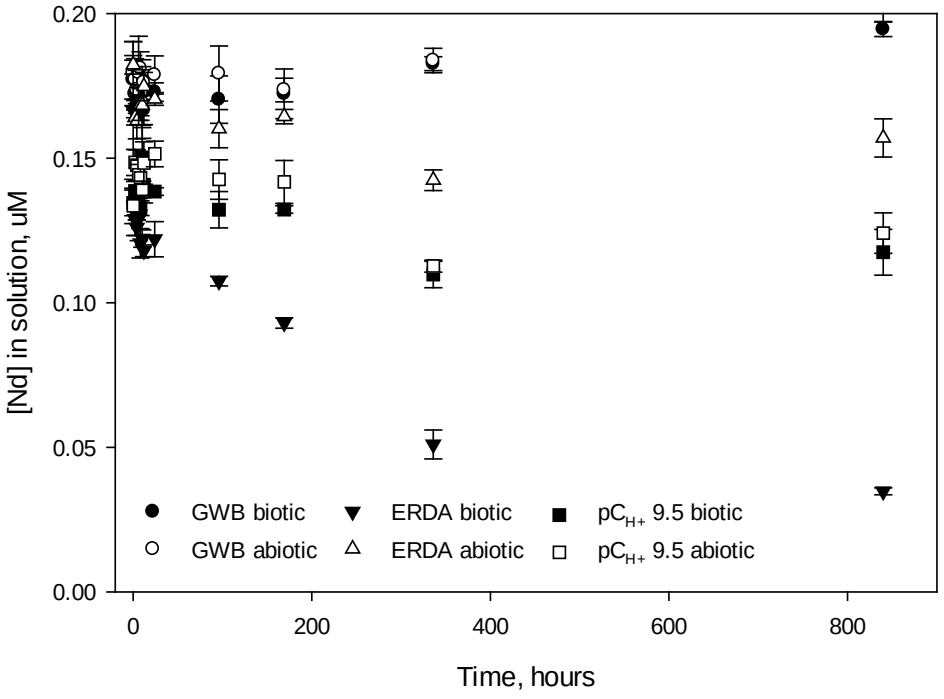



Figure 8. Neodymium in solution as a function of time in GWB, ERDA, and $pC_{H+}$ 9.5-specific brines, with and without *Halobacterium* sp. (GWB, 1.75 ± 0.52 x $10^9$ cells/ml; ERDA, 1.54 ± 0.23 x $10^9$ cells/ml; $pC_{H+}$ 9.5, 1.50 ± 0.20 x $10^9$ cells/ml).

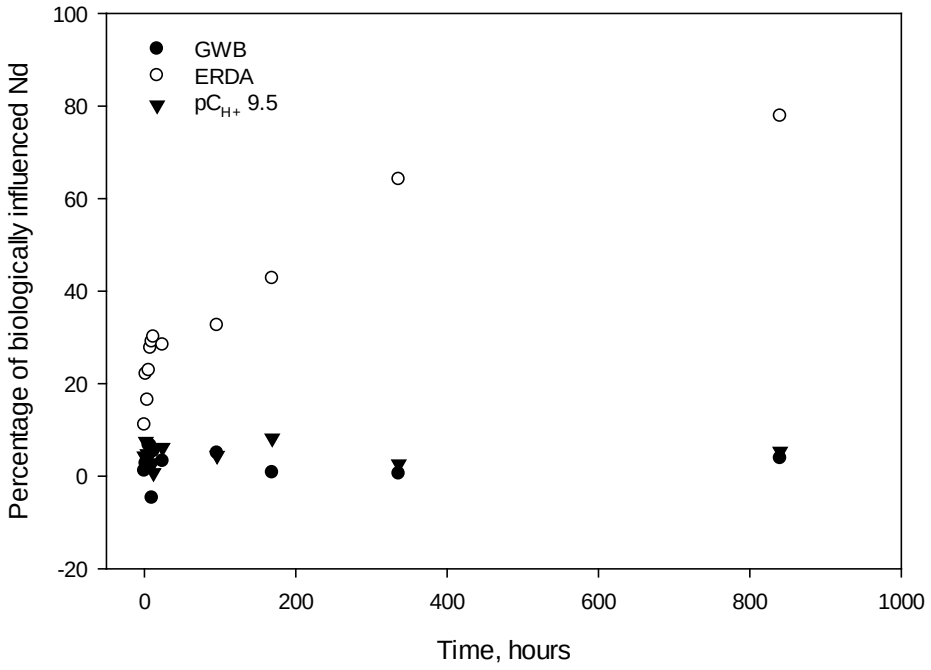



Figure 9. Percentage of biologically influenced Nd as a function of time in GWB, ERDA, and $pC_{H+}$ 9.5-specific brines.

**Influence of EDTA in WIPP brines**. A final experiment was conducted using Nd complexed with EDTA. The WIPP inventory-relevant concentration of $10^{-4}$ M EDTA was chosen. The presence of EDTA in solution prevented the loss of Nd from all three brines. (Figure 10).

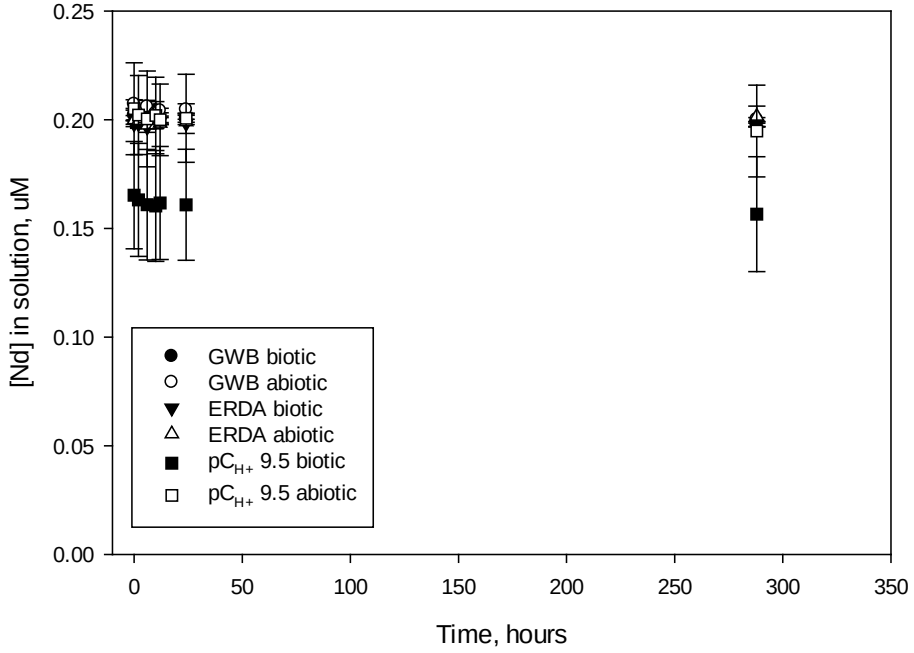



Figure 10. Neodymium in solution as a function of time in GWB, ERDA, and $pC_{H+}$ 9.5-specific brines, with and without *Halobacterium* sp., in the presence of EDTA.

In summary, these new data suggest that there are more processes involved with haloarchaea than simple surface complexation. The mechanisms of continued Nd loss from solution are still under investigation but could point towards 1) microbially-induced precipitation of the metal either at the cell surface or from solution or 2) internalization of the metal. If precipitation from solution is occurring, this can be considered beneficial to the WIPP as it lowers the source term. Internalization is of concern only if the organism is mobile. Bacterial results continue to suggest a fast sorption phenomenon that is ionic strength dependent and mitigated by the presence of EDTA.

## PART 5: CONCLUSIONS

It is the goal of this report to compile literature and experimental data that support the most realistic view possible for the biocolloid model. To this end, background information on the potential sources and possible numbers of microorganisms in the WIPP was presented; literature on actinide bioassociation at high ionic strength and with halophiles was reviewed; and new experimental data were also shown. The overall conclusions are as follows:

- Based on the microbial ecology of subterranean salt deposits and the bioenergetics of survival at high ionic strength, we submit that the majority of biomass present for the lifetime of the WIPP will be haloarchaea. Thus, bioassociation data for these organisms are most relevant. Bacteria that have been introduced with waste or other mining operations are unlikely to survive long-term, with the exception of extremely halophilic bacteria and spore-forming bacilli. These latter organisms require further investigation. The numbers of all these types of microbiota will be limited by the constraints of projected WIPP conditions (e.g., hypersalinity, low water activity, alkaline pH, anoxia, radioactivity).
- Bioassociation is affected by many factors, including ionic strength, different matrices, different $pC_{H+}$, and the presence of complexing ligands.
- Several studies with *Halobacterium* spp., including new experimental data, have found that there are more mechanisms involved in the long-term biological influence on actinides in solution than simple surface complexation. Complexation can occur with carboxyl and phosphoryl groups on S-layers or in EPS and can also lead to mineralization. These mechanisms do not necessarily lead to mobilization.

In summary, the concepts of PROPMIC and CAPMIC for determining a biocolloid contribution to the source term are logically sound. There must be some proportion of the actinides in solution that becomes associated with microorganisms (PROPMIC), and there must be some maximum concentration that can possibly associate (CAPMIC). However, experimental data used to generate these parameters can conservatively overestimate true mobile bioassociation, if they do not differentiate between mechanisms that result in immobilization and mobilization (e.g., mineralization). It is difficult to extrapolate from laboratory-scale experiments under reasonable time frames out to a repository's lifetime, especially when living organisms are involved and when the conditions for their growth or survival are suboptimal. Determining the nature of the interactions between WIPP microorganisms and emplaced actinides might help in predicting their permanence or their transience and will be the focus of future work.

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