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Credit author statement

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Biomass and salt-dependent effects of *Bacillus* spores on radionuclide migration from the Waste Isolation Pilot Plant

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ABSTRACT

Spores of a *Bacillus* sp., isolated from radioactive waste, were tested for their ability to influence the fate and transport of neodymium (Nd^{3+}) under high salt conditions expected at the Waste Isolation Pilot Plant (WIPP) nuclear waste repository. Spores were suspended in neodymium-spiked saline solutions up to 4 M NaCl, and concentrations of Nd and the complexing agent dipicolinic acid (DPA), a component of spores, were monitored along with optical densities and spore numbers. Results support neodymium bioassociation that is dependent upon biomass, with more apparent adsorption occurring at higher spore concentrations. However, probable spore lysis in 2 and 4 M NaCl solutions and possible germination at 0.15 M NaCl appear to drive the release of DPA and subsequent return of Nd to solution. The implications of this work for the WIPP will depend on actual biomass levels and the ionic strength of the repository brines.

Keywords: *Bacillus* spores, biological influence, neodymium, dipicolinic acid, WIPP

1. Introduction

The Waste Isolation Pilot Plant (WIPP) is the only existing terminal repository in the United States for transuranic waste. It is located ~650 meters below ground in a subterranean evaporite salt bed in southeastern New Mexico. The conditions of the salt bed (high ionic strength, low water activity, high concentrations of chaotropic solutes) combined with those projected for the repository itself (anoxia, radioactivity, uncertain nutrient supply) are not favorable for the growth, or even survival, of many microorganisms. However, *Bacillus* spores are resistant to many environmental extremes, including desiccation, heat, pressure,

radioactivity, and salt (Nicholson et al., 2000), which makes it likely that such spores could survive long-term in this environment and potentially interact with emplaced materials, including radionuclides.

In fact, members of the genus *Bacillus* have been detected in sediments contaminated with high-level nuclear waste, in plutonium-contaminated waste, in soils exposed to radioactive fallout from Chernobyl, in uranium waste piles, in WIPP-bound nuclear waste, and in a spent nuclear fuel pool (Fredrickson et al., 2004; Barnhart et al., 1980; Zavilgelsky et al., 1998; Selenska-Pobell et al., 1999; Swanson et al., 2015; Chicote et al., 2007). *Bacillus* species have also been isolated from salt mines worldwide, including from drift wall surfaces and air samples at the WIPP (Roohi et al., 2012; Carpa et al., 2014; Swanson et al., 2013; Swanson and Reed, 2018), and have been reported to remain viable within halite fluid inclusions for millions of years (Vreeland et al., 2000; Meng et al., 2015).

The potential effects of microorganisms on radionuclide mobility are well-known. Numerous studies have shown that spores can readily adsorb metals and even radionuclides—such as uranium, neptunium, strontium, and cesium—on their surfaces, often resulting in redox transformations (Revis et al., 1997; He and Tebo, 1998; Selenska-Pobell et al., 1999; Bargar, 2005; Carvajal et al., 2007; Allievi et al., 2011; Gorman-Lewis et al., 2013; Lujaniene et al., 2017) that can ultimately affect radionuclide fate and transport. As with other bacterial surfaces, sorption occurs due to the interaction of metals with anionic functional groups—such as carboxylate, phosphate, hydroxylate or amino groups—on the cell surface. In the case of spores, these groups are present in surface layer (S-layer) proteins, in the peptidoglycan of the cortex and core wall, and even in DNA and small proteins within the spore core (Ghosal et al., 2010; Allievi et al., 2011).

Another possible effect that spores might have on radionuclide mobility is through the release of the chelating compound 2,6-pyridine dicarboxylic acid, or dipicolinic acid (DPA). DPA is located within the spore core, where it can comprise a significant percentage of the core's dry weight (5-14%; Murrell, 1969; Setlow, 2003). Here, it immobilizes and protects essential macromolecules by forming a cross-linked "polymer gel" (Leuschner and Lillford, 2000; Jamroskovic et al., 2016). DPA is most often complexed 1:1 with Ca^{2+} but can also complex with other divalent cations, especially Mn^{2+} and Mg^{2+}. It is released from spores upon germination and can potentially complex with other cations, such as actinides, and enhance their mobility.

DPA has been proposed for use in the nuclear industry as an extractant during actinide separations, because it can form strong mono-, bis-, and tridentate complexes (see Table 1) with many lanthanides and actinides, as well as binary and ternary complexes (Heathman and Nash, 2012; Yusov et al., 2013; Jernstroem et al., 2007).

Table 1. Stability constants for An(III) and Ln(III) with DPA (Nd-citrate and Nd-EDTA are shown for comparison)

An/Ln-DPA	**log β_{101}**	**log β_{102}**	**log β_{103}**	**Reference**
Am	8.90 ± 0.02	15.87 ± 0.03	21.88 ± 0.04	Heathman and Nash, 2012
Eu	8.90 ± 0.02	15.64 ± 0.03	21.65 ± 0.04	Heathman and Nash, 2012
La	7.94	13.71	17.95	Jernstroem et al., 2007
Ce	8.29	14.33	18.67	Jernstroem et al., 2007

Nd	8.73	15.40	20.41	Jernstroem et al., 2007
Nd-cit	6.94 ± 0.03	10.91 ± 0.01	---	Brown et al., 2014
Nd-EDTA	16.51	---	---	Jagoda et al., 2018

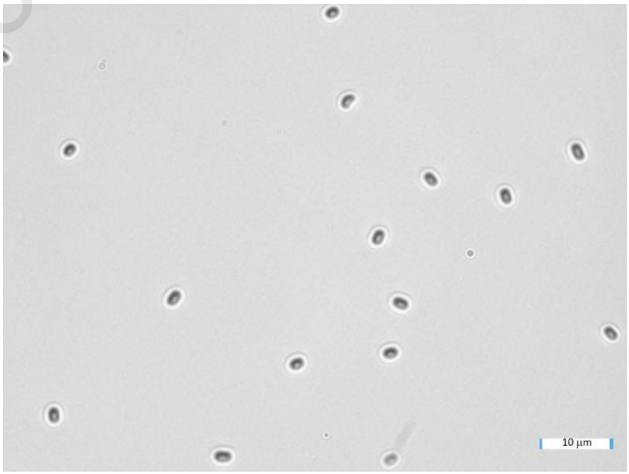
To date, there are no published data on metal/actinide sorption onto bacterial spores at ionic strengths higher than 0.5 M. Further, the majority of experiments below 0.5 M were conducted in perchlorate solutions. By comparison, WIPP brines are high in ionic strength, especially NaCl (Na^+ of 3.5-4.9 M), and it is possible that chloride will influence spore germination (Rode and Foster, 1962; Nagler et al., 2014; Nagler and Moeller, 2015). Here, we present findings from experiments to determine if spores of a *Bacillus* species present in WIPP-bound waste could influence the dissolved concentration of the lanthanide, neodymium, via biosorption or DPA release at high salt concentrations. The non-radioactive analog, neodymium(III), was chosen for these experiments to represent the +3 actinide oxidation state.

2. Materials and methods

2.1. Organism preparation and spore purification

Bacillus sp. strain WW-6-2 was isolated from the contents of a WIPP-bound transuranic waste drum (Swanson et al., 2015). It is not halophilic, thus it is unlikely to be in a vegetative state in a brine-filled repository (Figure S1). For these experiments, cells were initially grown in R2B (containing in g/L: yeast extract, 0.5; proteose peptone #3, 0.5; casamino acids, 0.5; dextrose, 0.5; soluble starch, 0.5; sodium pyruvate, 0.3; K_2HPO_4, 0.3; $MgSO_4 \cdot 7H_2O$, 0.05), then plated onto modified Schaeffer's sporulation agar (containing in g/L: nutrient agar, 16;

81 $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 2.0; $Ca(NO_3)_2 \cdot 4H_2O$, 0.236; $MnCl_2 \cdot 4H_2O$, 0.02; glucose, 1;
82 $FeSO_4 \cdot 7H_2O$, 0.1 ml of 10 mM solution; Leighton and Doi, 1972; Smith et al., 2011) and
83 incubated for a minimum of 1 week at 28°C. Plates were flooded with 10 ml of sterile high
84 purity water (HPW; 18.2 MΩ·cm) and allowed to sit for 10 minutes. Using a sterile spreader,
85 cells were gently scraped from the agar surface and transferred to a sterile centrifuge tube. In
86 some experiments, spore purification involved an initial incubation with lysozyme (50 µg/ml) in
87 a 37°C water bath for 1 hour with occasional vortexing, followed by a series of 4-5 washes in
88 high purity water (HPW). Because of the possibility that lysozyme compromised spore coats,
89 the lysozyme step was omitted from other experiments, and spores were only washed with HPW.
90 In both cases, the final wash was divided evenly into three separate tubes, and the spore pellets
91 were stored overnight at 4°C prior to the experiment. Spore purification was determined
92 microscopically, by surveying at least 50 fields for the presence of vegetative cells, using a Zeiss
93 Axioscope 40 (Zeiss Microscopy; Pleasanton, CA). If any vegetative cells were observed during
94 the survey, the experiments were not initiated.



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96 Figure 1. Bright field image of spores prior to start of experiment; image is representative of
97 surveyed fields demonstrating spore purity.

2.2. Reagents

Three salt concentrations were tested in these experiments: 0.15M NaCl ("normal physiological saline"), 2M NaCl, and 4M NaCl. Each solution pH was adjusted to approximately 5.0 in order to maintain neodymium solubility. Parent Nd stock solutions with higher concentrations were prepared by adding $NdCl_3 \cdot 6H_2O$ (Aldrich Chemical Company; 99.9% purity) to each salt solution and adjusting pH if necessary. Neodymium in these concentrated stock solutions was measured to verify starting concentration (details in section 2.5). These solutions were then used to spike the test solutions to a final concentration of approximately 2 µM. All solutions were passed through a 0.22 micron nylon filter prior to use.

2.3. Nd sorption kinetics experiments

Spore pellets purified with lysozyme were resuspended in each target matrix (0.15 M, 2M, and 4M NaCl) to obtain a "HIGH" biomass concentration (equivalent to approximately 10^8 spores/ml, as determined by optical density, OD) and a "LOW" biomass concentration (equivalent to approximately 10^6 spores/ml). Spore pellets that were not purified with lysozyme were resuspended to obtain three biomass concentrations: high (~10^8 spores/ml), mid (~10^7 spores/ml), and low (~10^6 spores/ml). All suspensions at each salt concentration were then mixed 1:1 with the same salt solution that contained 2 µM neodymium to achieve a final [Nd] of ~1 µM. Samples were withdrawn from each suspension at multiple time points following mixing. These were measured for optical density on a GeneSys 50 UV-Vis spectrophotometer (ThermoFisher Scientific; Waltham, MA) prior to passing through a 100 kD centrifugal filter. Aliquots of the filtrate were diluted in either 2% nitric acid for mass spectrometry analysis or in 25 mM nitric acid for DPA analysis by ion chromatography (additional measurement details

provided below). Replicate filtrates for each sample set were pooled to measure pH. Periodic spore counts were performed by microscopy.

2.4. Nd-free DPA tracking experiment

DPA concentrations were tracked during spore purification procedures with and without lysozyme (Figure S2) and during a mock kinetics experiment without Nd or lysozyme. Four biomass concentrations were used: ~10^8 spores/ml; ~10^7 spores/ml; ~10^6 spores/ml, and ~10^5 spores/ml. Replicate samples were withdrawn at various timepoints and were passed through a 100 kD filter and diluted in 25 mM nitric acid for analysis by ion chromatography. Periodic direct microscopic counts were made on the 10^6-10^8 spores/ml samples (high, mid, low); while, the 10^5 spores/ml samples were plated onto R2A for colony-forming unit (CFU) counts, as these were mostly below the microscopic detection limit.

2.5. Inductively coupled plasma-mass spectrometry (ICP-MS) analysis

All Nd measurements were determined using an Agilent 7900 ICP-MS (Santa Clara, CA) equipped with an Agilent ASX-500 series auto-sampler. Calibration of the ICP-MS against prepared Nd solutions, from a commercial standard (1000 μg/ml in 2% nitric acid; High Purity Standards; Charleston, SC), consistently employed at least four points of response measurement, and resultant linearity values were frequently better than 0.999. Indium was utilized as an internal standard throughout all measurements via direct addition to each sample from the same stock solution, prepared from a 1000 μg/ml standard in 2% nitric acid (High Purity Standards).

2.6. DPA analysis by ion chromatography

DPA was analyzed on a Dionex ICS-6000 ion chromatography system (ThermoFisher Scientific; Waltham, MA) equipped with a UV detection system using an IonPac AS7 column

with corresponding AG7 guard column, 25 mM nitric acid eluent at an isocratic flow rate of 0.5 ml/min and with UV detection set at 290 nm. System control and data acquisition were managed using the vendor's Chromeleon 7 Chromatography Data System (V 7.2.8). Calibrant solutions were prepared by dilution from a 1000 μg/ml solution, of DPA (Acros Organics; 99% purity) dissolved in high purity water. Replicate blank and calibrant solution check measurements were performed at least every ten samples to verify continued system functioning.

2.7. Nd speciation

Neodymium speciation in the three test systems was calculated using the EQ3/6 v8.0a modeling package, equilibrated to atmospheric CO_2/O_2, and using values derived from the YPF revision 2 database (Wolery and Daveler, 1992). pH values were constrained to between 5 and 6.

3. Results and discussion

3.1. Establishing Nd speciation in experimental matrices

Preliminary calculations of Nd speciation in the experimental solutions were carried out using the EQ3/6 modeling package, as described above, to ensure analyte availability in the experiments. Calculated results for neodymium speciation in the experimental systems used in this work are shown in Table 2. At all salt concentrations, more than 95% of Nd is expected to be present as the free ion, with the mono-chloride species representing the second largest contributor to total species. Thus, the majority of Nd was available for interaction in solution.

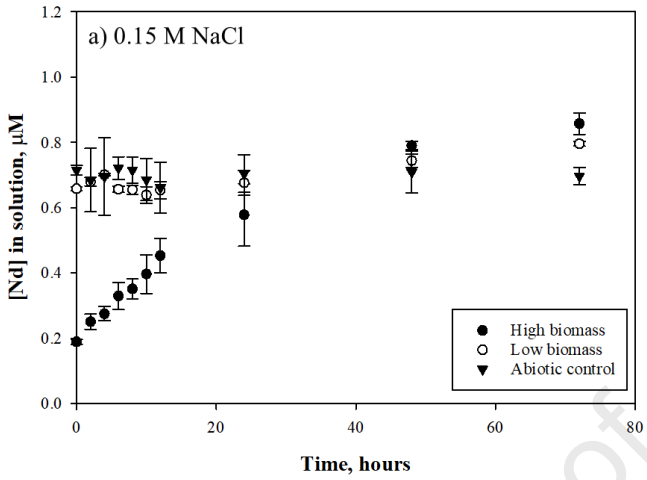
Table 2. Neodymium speciation (as percent) in experimental solutions of varying NaCl concentration.

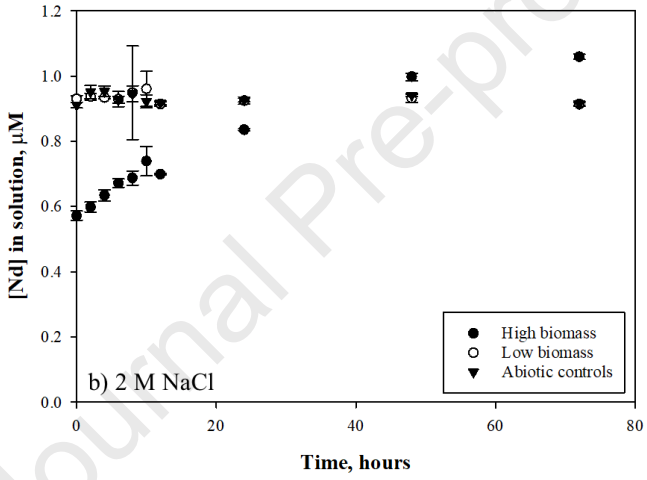
Species	0.15 M	2 M	4 M
Nd^{3+}	96.53	97.31	97.92
$NdCl^{2+}$	2.92	2.54	1.64
$Nd(OH)^{2+}$	< 0.5	< 0.5	< 0.5

3.2. Nd kinetics

3.2.1. Nd kinetics after spore purification with lysozyme

Nd interactions with lysozyme-purified *Bacillus* spores were investigated over a 72-hour period, using three biomass concentrations at three salt concentrations, along with abiotic controls. The concentration of Nd in solution in the abiotic controls and in the low biomass samples remained relatively stable over time at all tested salt concentrations and did not appear to differ from each other. In contrast, Nd levels in the high biomass sample filtrates were significantly lower than the abiotic controls at the initial time point but then increased with time, such that all the added Nd was back in solution after 72 hours (see Figure 2). The pH values remained consistent over time and did not differ from the abiotic control.





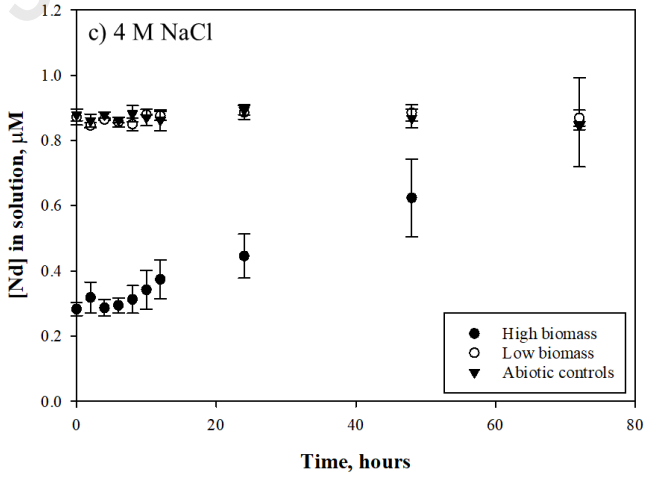


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Figure 2. Neodymium measured in solution over time, in the presence and absence of *Bacillus* spores (purified with lysozyme step) and at three different NaCl concentrations a) 0.15 M, b) 2 M, and c) 4 M. Error bars represent one standard deviation from the mean of triplicate samples.

The mechanism of biological influence at time zero was most likely rapid sorption onto available sites at the spores' surfaces. This influence varied between each salt concentration but did not correlate with the concentrations of NaCl used: 75% at 0.15 M, 37% at 2 M, and 68% at 4 M. In contrast, the rate at which Nd returned to solution was highly dependent on salt concentration, with the highest rate of resolubilization observed at the lowest salt concentration (Table 3).

Table 3. Rates of Nd resolubilization in high biomass samples (over first 24 hours)

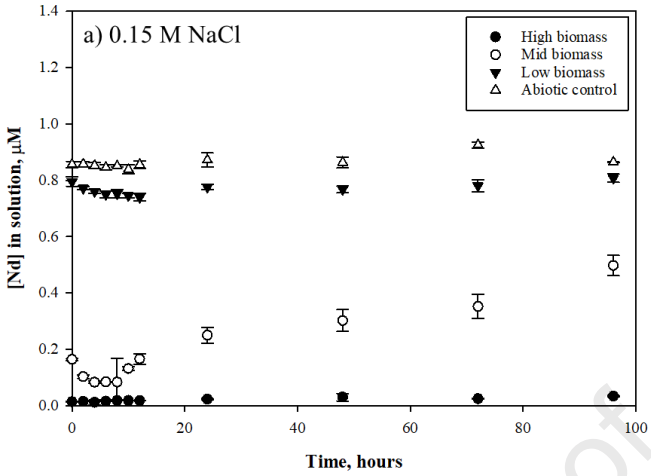
[NaCl]	Rate (nM/hr)
0.15 M	16.2
2 M	11.0
4 M	6.79

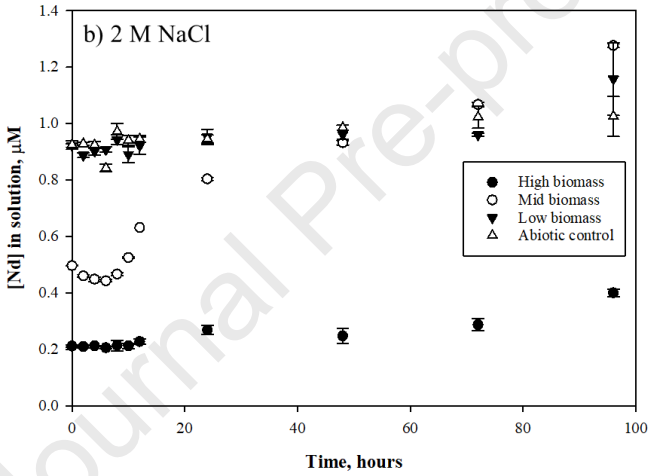
These observations are not readily explained by any one mechanism and likely point to more than one type of influential interaction in these experimental systems.

3.2.2. Nd kinetics after spore purification without lysozyme

Analogous investigations of Nd kinetics were carried out following purification of the *Bacillus* spores without the lysozyme step, to determine if this step affected previous

observations. As shown in Figure 3, results from experiments at low biomass were comparable to those shown above and yielded little Nd loss from solution over the course of the experiment, relative to the abiotic control. However, in the high biomass samples, almost all Nd immediately associated with the spores and remained sorbed throughout the course of the experiment, instead of resolubilizing as was observed with lysozyme-purified spores. In the mid-biomass samples (~10^7 spores/ml, which were added to capture effects in the gap between high and low), the concentration of Nd in solution initially decreased but then began a slow increase with time. This could suggest a competitive interaction between surface sorption sites on the spores and something in solution. The fact that the rate of Nd resolublization at 0.15 M NaCl in these experiments was much slower than in the previous set of experiments (6.71 nM/hr versus 16.2 nM/hr) while the rate at 2 M (11.6 nM/hr) is similar, also suggests that the mechanism of resolubilization is different at low versus high salt concentrations. In the low biomass samples, very little neodymium associated with biomass over time: approximately 10% at 0.15 M NaCl and no more than 7% at either 2 or 4 M. pH values remained consistent over time.





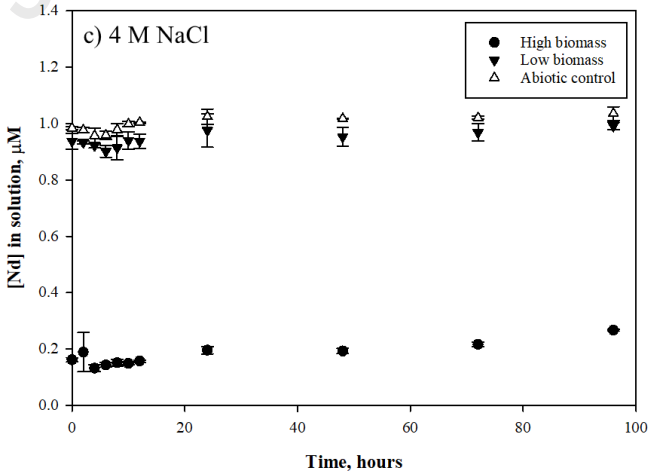


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Figure 3. Neodymium measured in solution over time, in the presence and absence of *Bacillus* spores, at three different salt concentrations (lysozyme-free experiments): a) 0.15 M, b) 2 M, and c) 4 M. Error bars represent one standard deviation from the mean.

3.3. DPA release during Nd kinetics experiments

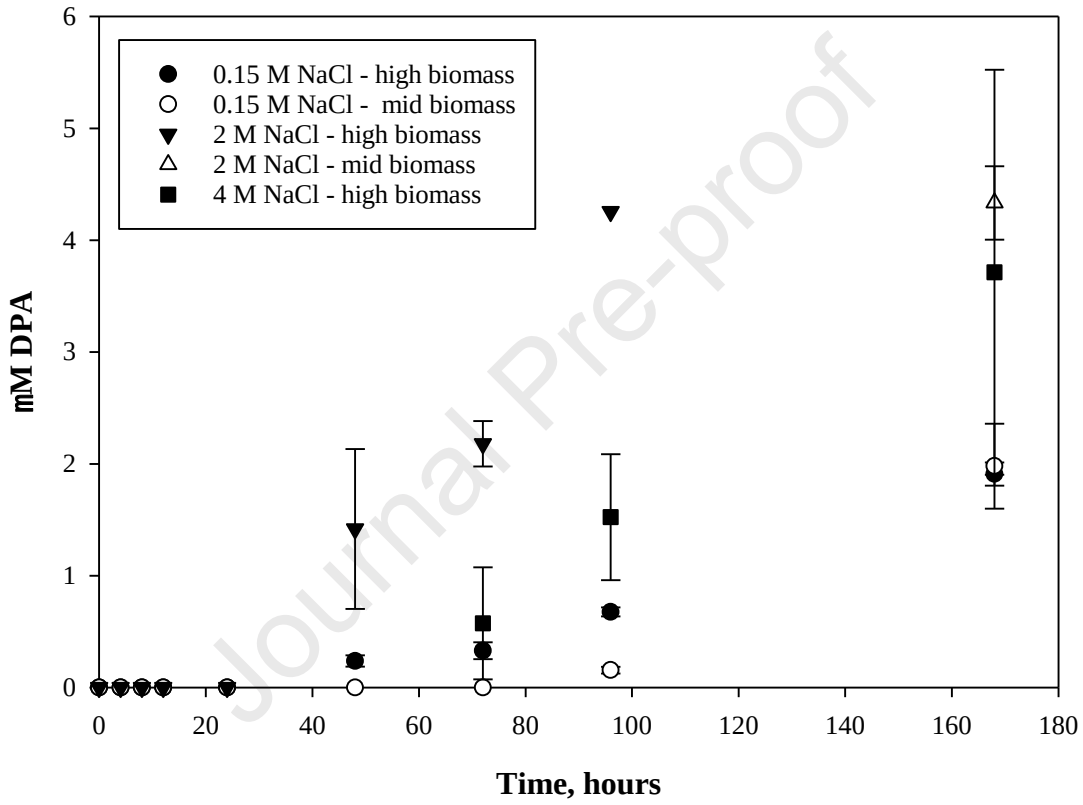
3.3.1. DPA release after spore purification with lysozyme

All kinetics samples were analyzed for DPA by ion chromatography to determine if its release was the possible cause of Nd resolubilization. No DPA was detected during the course of the original kinetics experiments with Nd and lysozyme. This could have been due to a couple of factors. First, samples were significantly diluted to accommodate the instrumentation. This could have caused the level of DPA in the samples to drop below the detection limit (~0.1 mg/l). Second, the Nd-DPA complex may not have been readily detectable by UV spectroscopy due to significant signal quenching upon formation of the complexed species (see Figure S3). The conventional method of measuring DPA is with terbium fluorescence. However, given that this study revolves around lanthanide/actinide interactions, this method was not feasible. Similar detection issues also prompted Gorman-Lewis et al. (2013) to use actinide-free experiments to track DPA in their samples.

3.3.2. DPA release after spore purification without lysozyme

In Nd kinetics experiments without lysozyme, DPA was not detected until later time points, if at all, and did not correlate with salt concentration (Figure 4). These samples were not diluted prior to analysis by IC, but frequent rinse steps were incorporated into the analysis. In the high biomass samples, DPA appeared between 48-72 hours; in the mid biomass samples,

231 DPA appeared later at 96 hours at 0.15 M and at one week in 2 M. No DPA appeared in any low
232 biomass samples over the time course of the experiments. The late appearance of DPA could be
233 a reflection of the Nd-DPA ratio required for UV detection and suggests an underestimation of
234 the actual DPA released (see Figure S3).



235

236 Figure 4. DPA measured during Nd kinetics experiment without lysozyme.

237

238 *3.4. DPA release during mock kinetics experiment without Nd*

239 To test whether DPA had, in fact, been released during the Nd kinetics experiments with
240 lysozyme but was simply not detected, a series of mock experiments was run without Nd.

241 Samples were not diluted prior to analysis by IC. These mock experiments were also run using
242 spores purified with and without lysozyme, and samples were withdrawn during both
243 purification procedures to analyze for DPA.

244 *3.4.1. Without lysozyme*

245 Almost no DPA was released during the spore purification procedure without lysozyme,
246 but a rapid release of low levels of DPA was observed upon spore resuspension in the salt
247 solutions (Figure 5). At 0.15 M NaCl with high biomass, the amount released increased
248 gradually over time, reaching up to 3.4 µM at one week. At mid-level biomass concentrations,
249 the release was minimal and sporadic with no temporal correlation. There was no detectable
250 release from the low biomass samples. At 2 M NaCl, there was a slight increase in measured
251 DPA at the later time points and a slight, but not significant, biomass correlation. By 1 week,
252 these samples had released between 2.3-3.3 µM DPA. At 4 M NaCl, there was neither a change
253 in DPA concentration with time nor a biomass correlation, and the amount of DPA released
254 (mostly < 1 µM) was less than in the lower salt concentrations.

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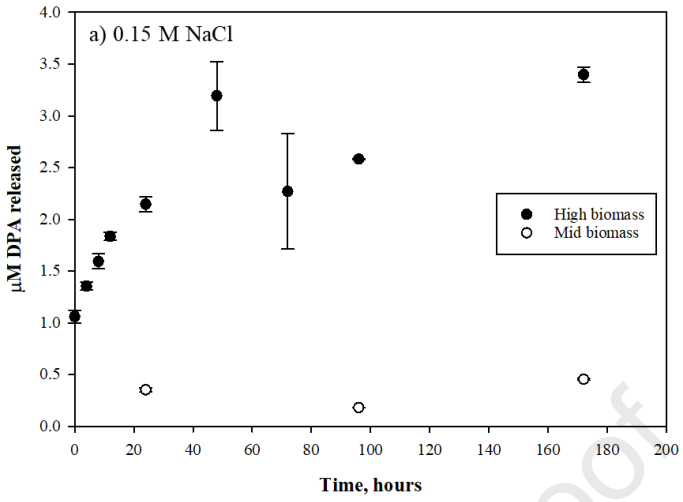
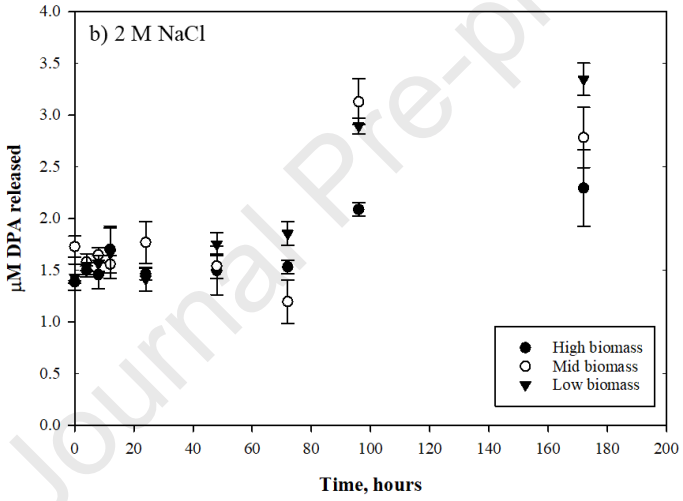
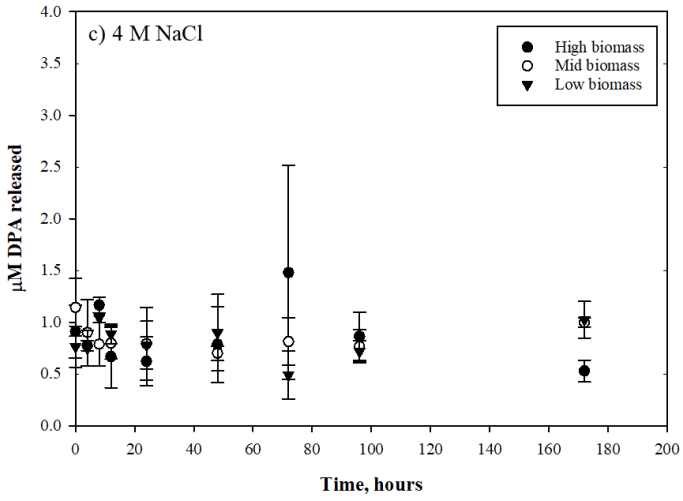






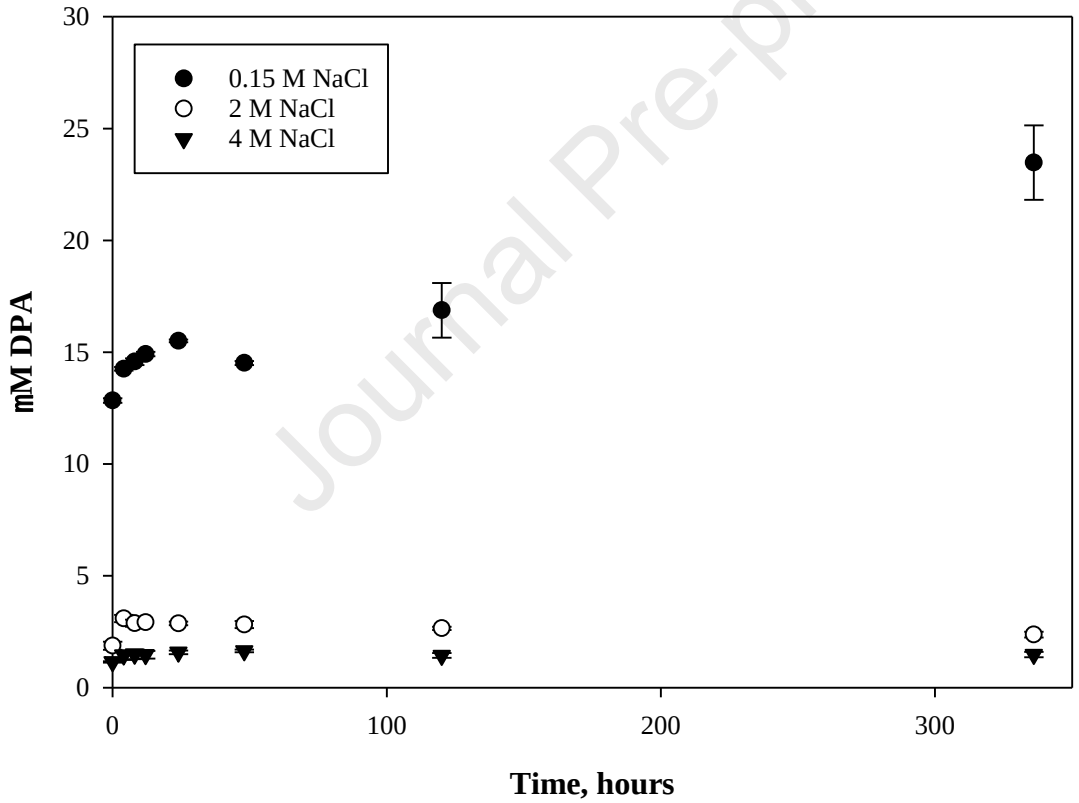
256

257 Figure 5. DPA measured during Nd-free/lysozyme-free mock kinetics experiment.

258

259 *3.4.2. With lysozyme*

260 When lysozyme was used during spore purification, the levels of DPA released in the 2
261 and 4 M samples were within the same range as without lysozyme but were much higher in the
262 0.15 M samples (Figure 6).



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264 Figure 6. DPA measured during Nd-free mock kinetics experiment with lysozyme, high biomass
265 only.

266

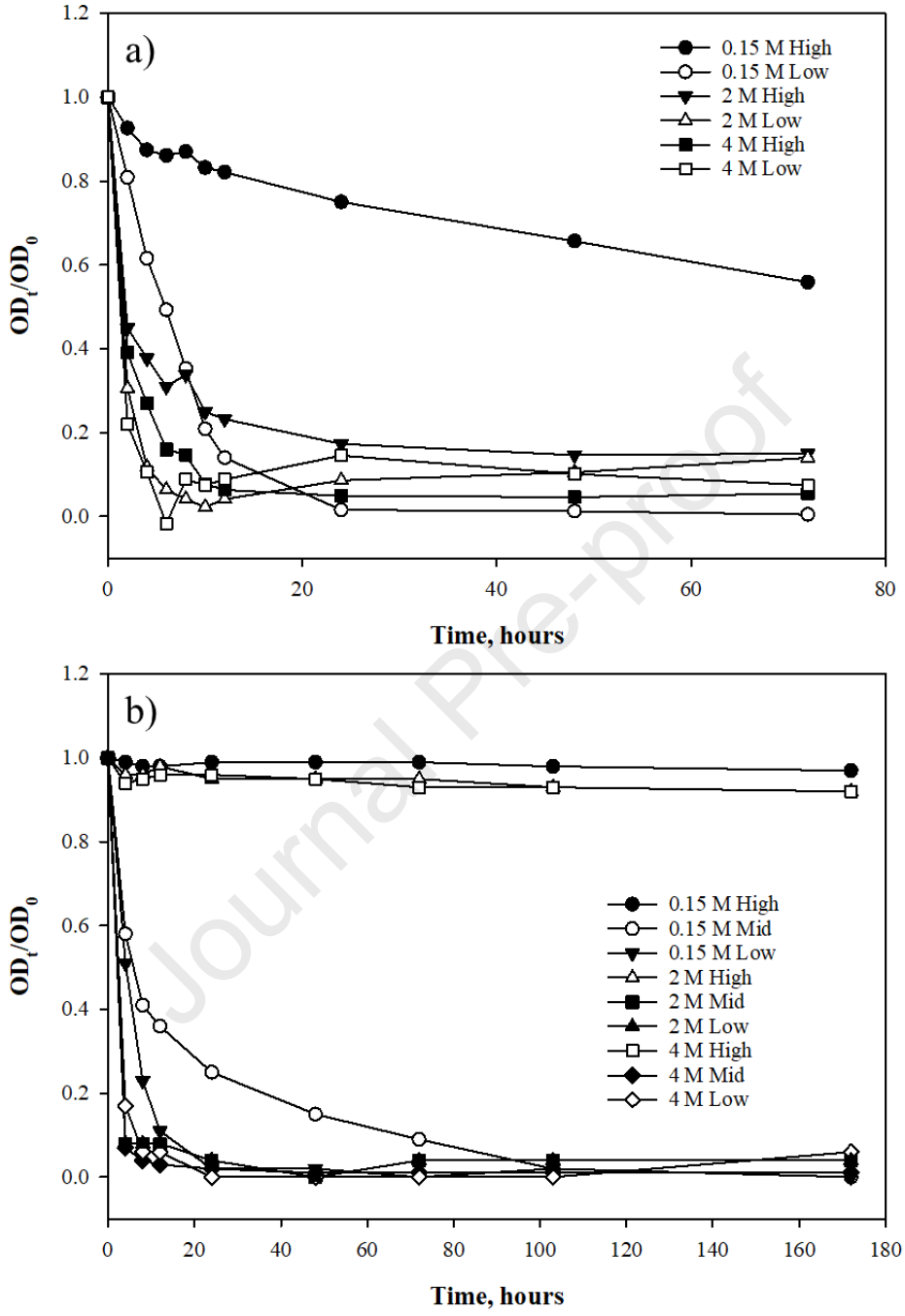
The inhibition of germination and the subsequent release of DPA at increasing salt concentrations has been shown for several *Bacillus* species (Nagler et al., 2014, 2016; Nagler and Moeller, 2015). These results agree with their findings. However, in the Nagler studies, a nutrient germinant was always added; whereas in this study, there were no compounds present that should have led to DPA release via germination, suggesting that there was another cause of DPA release. Given the differences in results between lysozyme and lysozyme-free experiments, it was hypothesized that lysozyme also caused some compromise in spore coat integrity that enhanced DPA release at 0.15 M NaCl. Results from the two different purification procedures (with and without lysozyme) suggest that this may have been the case (Figure S2). Nevertheless, DPA release still occurred in samples that had not been purified with lysozyme (Figures 4 and 5).

Lysozyme is often used in spore purification protocols to ensure the elimination of vegetative cells. Most *Bacillus* spores are resistant to lysozyme, except in cases where the outer membrane and spore coat have already been compromised, such as with UV irradiation or treatment with surfactants (Setlow, 2003, 2014; Gould and Hitchins, 1968). Otherwise, lysozyme is presumed to be too large to penetrate through the spore coat (Setlow, 2014). However, some *Bacillus* spp. are susceptible to lysozyme, and this could be the case with the test organism here.

3.5. Effects on biomass

Biomass concentrations were tracked using both optical density and direct microscopic counts. In experiments that used lysozyme, there was a decrease in the OD of all samples over time (Figure 7a). In experiments without lysozyme, this same trend was observed but not at high biomass concentrations (Figure 7b). The decrease in ODs correlated with both salt concentration

290 and biomass concentration: the rate of decrease in OD was greatest at higher salt concentrations
291 and at lower biomass. The greatest decrease occurred at 2 M and 4 M NaCl. The same was
292 observed for the low biomass suspensions, but the difference between the 2 and 4 M samples
293 was often negligible.



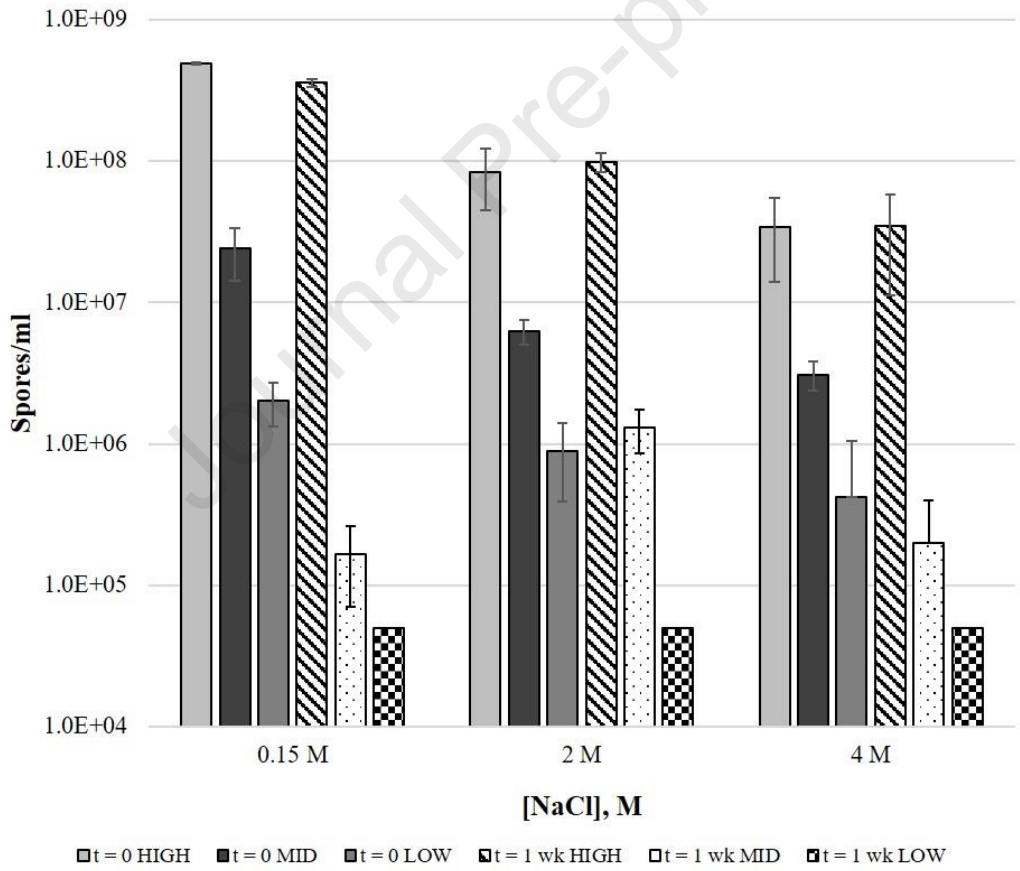
294

295 Figure 7. Optical density of each sample relative to its original optical density in experiments

296 with (a) and without (b) lysozyme during spore purification.

297

298 Spore numbers from direct microscopic counts also decreased over the course of the
299 experiments (Figure 8). The decrease was more dramatic for the low biomass samples but also
300 for the high biomass samples at 4 M NaCl. In some of the 2 and 4 M NaCl-low biomass
301 samples, counts dropped below the detection limit by microscopy in as little as 24 hours. In the
302 4 M-high biomass samples, an increase in spore counts was occasionally observed. This may
303 have been due to clumps formed at the beginning of the experiments that may have dispersed as
304 time went on. This could also account for the observed initial decreases in relative optical
305 densities.



306

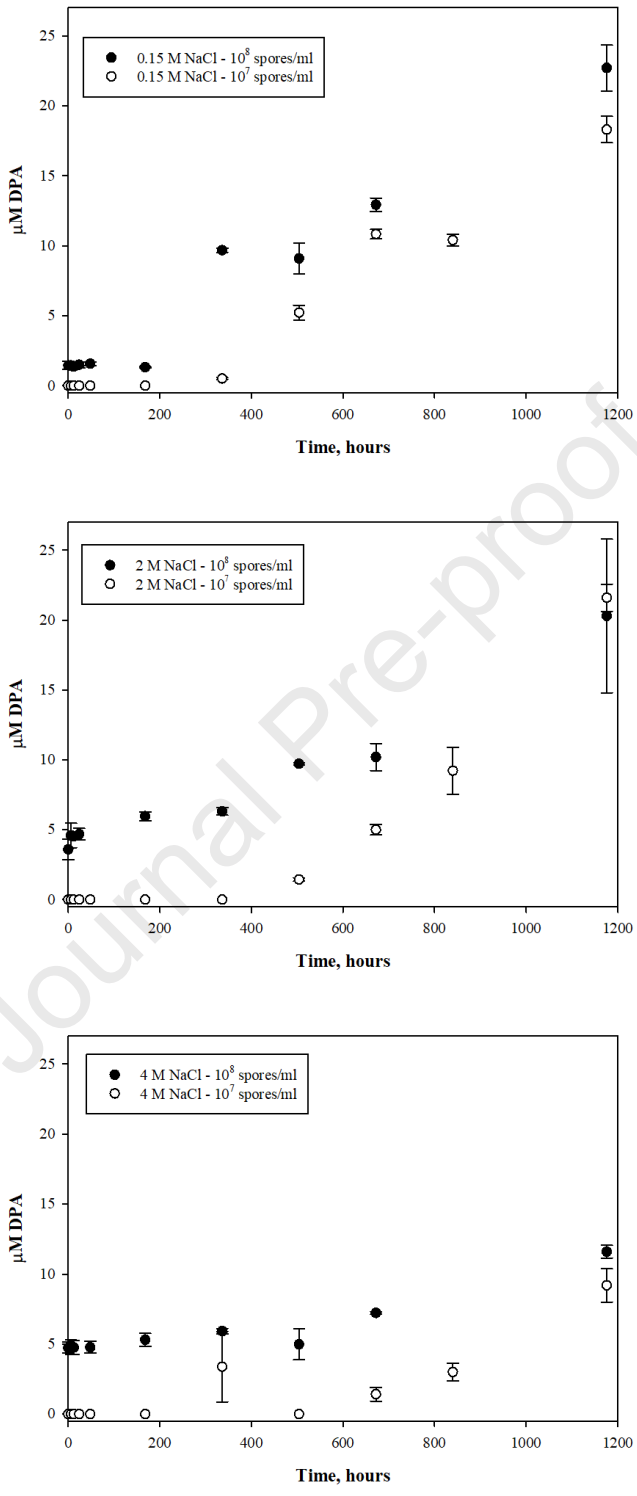
307 Figure 8. Spore counts at t = 0 and after 1 week in lysozyme-free/Nd-free kinetics experiments.
308 Error bars represent the standard deviation from the mean, except in the 0.15 M high biomass

sample at one week, where the bar represents the range of two replicates. No spores were observed in 4 M-low biomass samples; therefore, a detection limit of 5.00E+04 spores/ml is graphed for comparison.

Changes in optical density are often used as measures of spore germination because of the non-refractile nature of germinating spores (Powell, 1950; Fleming and Ordal, 1964). The decrease in OD observed in these experiments, combined with the decrease in spore numbers by microscopic counts, suggests that either the spores began to germinate, and in doing so, were promptly lysed by the high salt concentrations or that spores simply lysed immediately upon exposure, due to osmotic effects (Figures 7 and 8). Rode and Foster (1962) showed that germination could be stimulated by low (~0.1-0.2 M) concentrations of various salts—including NaCl, KCl, and $MgCl_2$ –and this could have been the case in the 0.15 M samples, even though there were no nutrient germinants present. Spot checks using phase contrast microscopy did not support consistent, if any, germination, although it should be noted that checks could not be performed immediately upon resuspension. Moreover, although each purified spore pellet contained roughly the same wet weight of biomass prior to resuspension, the initial ODs upon resuspension differed significantly with salt concentration (e.g., 0.907 at 0.15 M NaCl, 0.524 at 2 M NaCl, and 0.311 at 4 M NaCl), suggesting that higher salt had a drastic and immediate effect on spore numbers. Therefore, the most likely cause of DPA release in these experiments was simple lysis due to osmotic stress. The use of lysozyme during spore purification may have decreased the time-to-lysis by compromising the spore coats, but it was not likely the proximate cause.

We also hypothesize that the high salt concentrations led to spore coat compromise and subsequent lysis at lower biomass concentrations where spores were more exposed to the bulk solution. This is supported by the pronounced decrease from 10^6 spores/ml to below the microscopic detection limit in low biomass samples, and was especially evident at 2 and 4 M NaCl (Figure 8). In the biomass-dependent experiments, the 10^5 spores/ml samples were plated for CFU counts. All 2 and 4 M NaCl samples dropped from 10^5 to mid 10^3 CFU/ml within 6 hours and remained at that level for weeks, while the 0.15 M NaCl samples fell to 10^3 at 48 hours.

DPA release was dependent upon biomass concentration, with the highest releases occurring at the highest biomass. More DPA was released in the 0.15 M samples, regardless of biomass; however, the difference between DPA released in the 0.15 M and 2 M samples was not always significant (Figure 9).



343

344 Figure 9. DPA release from 10^8 spores/ml and 10^7 spores/ml samples in biomass dependence

345 experiments. No DPA was detected in the 10^5 spores/ml samples over the time course of the

346 experiments. A small amount of DPA (0.35 µM) was released in the 10^6 spores/ml samples at
347 0.15 M NaCl at the last time point but is not shown here.

348

349 **4.0. Conclusions**

350 *4.1. Experimental conclusions*

351 These experiments did not demonstrate biosorption of Nd onto *Bacillus* spores under
352 most conditions tested. Instead, we observed a steady return of Nd back into solution and a
353 "concomitant" release of DPA in parallel Nd-free experiments (but also a delayed measurable
354 release of DPA in experiments with Nd). This resolubilization of Nd occurred in experiments
355 both with and without lysozyme, but the rate was much slower in experiments without lysozyme.

356 In all experiments, regardless of lysozyme usage, the fate of Nd was dependent upon
357 biomass concentration and salt concentration. At high biomass concentrations, Nd remained
358 adsorbed to spores for a longer period of time (at least 96 hours) before slowly returning to
359 solution. High biomass samples released more DPA overall, but this release was significantly
360 lower at 4M than at 0.15M. It is possible that high biomass had a protective effect against salt
361 exposure, such that spore integrity was not compromised as quickly as in lower biomass samples,
362 especially at 2 and 4 M.

363 Harrold and Gorman-Lewis (2013) also found that DPA release was biomass
364 concentration dependent, with up to five times more DPA released per kg of wet spore weight
365 when biomass was high. We could not normalize our results to spore wet weights in this study,
366 since they varied with salt concentration as fluids penetrated into the spores. This uptake is
367 likely what led to spore lysis and DPA release.

Based on experiments by Rode and Foster (1962) and Nagler et al. (2014), spores may have germinated at 0.15 M NaCl, accounting for higher DPA release and more Nd resolubilization. Although Nagler and Moeller (2015) did observe 88% germination of their spore population at 2.4 M NaCl, this was in the presence of a nutrient germinant. In our case where no nutrients were present, it is unlikely that spores germinated at either 2 or 4 M, suggesting that the release of DPA occurred upon lysis. In either case, the fate of Nd at the lower biomass concentrations was tied to the osmotic effects of salt on spores.

Metal desorption from spores has been documented previously. In experiments with a marine *Bacillus* strain, copper(II) sorbed rapidly onto spores but subsequently desorbed, except when the spores were fixed with glutaraldehyde or where the experiments were run at 4°C (He and Tebo, 1998). The use of a fixative or exposure to low temperature would have prevented germination and subsequent DPA release. In investigations of neptunium adsorption onto *B. subtilis* spores, progressive desorption over time was observed at elevated pH (Gorman-Lewis et al. 2013; Harrold and Gorman-Lewis, 2013). This was attributed to the competition for Np complexation between surface sites and DPA in solution.

4.2. Fate of Bacillus spores and biologically influenced An(III) in the WIPP Environment

Bacillus spores have undoubtedly been introduced into the WIPP on equipment and personnel throughout the operations phase and have been introduced with the waste itself. It is assumed that the highest concentration of spores has been introduced with soil wastes. The current WIPP inventory records 5.75×10^6 kg of soil in WIPP waste (van Soest, 2018). This translates to approximately 330 grams of soil per liter of possible intruding brine (US DOE CRA 2014, Appendix MASS). A previous study of contaminated soil from a transuranic waste burial site found between 10^4-10^6 CFU per gram, depending on depth and radiation levels (Barnhart et

al., 1980). Assuming all these colonies derived from spore-forming organisms (a possible overestimate) and assuming waste soils contain similar numbers, this would yield between 3.3 x 10^3 and 3.3 x 10^5 spores/ml of intruding brine. This number falls in the "low" biomass concentrations used for this study, where neither sorptive nor solubilizing effects were seen. If a high number of spores is assumed (e.g., 5 x 10^8 spores/g soil), based on literature values from a range of uncontaminated soils (Amann et al, 2011), then approximately 1.65 x 10^8 spores/ml of brine can be predicted. This latter value is close to the "high" biomass designations used in these experiments. Based on our data, the fate of spores at mid-level biomass (~10^7 spores/ml) concentrations is likely to be lysis and DPA release, leading to solubilization and mobility of An(III). At higher biomass numbers, spores may initially remain intact and adsorb actinides, leading to a lower concentration of in solution with DPA. Nevertheless, even in the high biomass samples, DPA was gradually released and could contribute to actinide solubilization.

The findings of this study are based on a single *Bacillus* isolate whose spores appear to be susceptible to lysozyme, unlike most *Bacillus* spores. The amount of DPA present in *Bacillus* spores and the resistance of those spores tends to vary significantly and is not necessarily biomass dependent or species dependent but may depend more on the conditions under which sporulation occurred (Bressuire-Isoard et al., 2018). For example, significantly less DPA was synthesized and released from *B. subtilis* spores formed at lower temperatures (i.e., 25 versus 42 °C; Isticato et al., 2019). Sporulation conditions for WIPP waste bacilli are impossible to know.

There are likely to be other conditions at WIPP and in WIPP wastes that will affect bacillus sporulation and spore integrity besides salt concentration. Constituents of WIPP brines may have different effects on germination. In the studies by Nagler and Moeller (2015), magnesium chloride strongly inhibited germination in several *Bacillus* species at 1.2 M; WIPP

414 brines contain up to 1 M magnesium chloride. Potassium chloride is a weak inhibitor of
415 germination at 0.6 M; WIPP brine concentrations reach 0.47 M. High ionic strength conditions
416 may also lead to the lysis of non-halotolerant microorganisms, resulting in the release of nutrient
417 germinants, for example alanine present in cell walls.

418 Constituents of WIPP wastes are also likely to affect spore germination. For example,
419 nitrate salts have been shown to stimulate germination at low concentrations but strongly inhibit
420 germination at the projected WIPP concentration (Rode and Foster, 1962; Nagler and Moeller,
421 2015). Phosphates moderately inhibit germination at the projected WIPP inventory
422 concentration. Organic waste components, such as the complexants acetate and citrate, had no
423 effect on germination at 0.1 M, which is higher than the projected inventory for those organics in
424 WIPP (Rode and Foster, 1962).

425 Because the WIPP receives only low-to-intermediate level nuclear waste, ionizing
426 radiation is unlikely to affect spore viability. However, some wastes may generate heat
427 sufficient to activate spore germination, which can occur at temperatures as high as 78°C
428 (Levinson and Hyatt, 1969).

429 Finally, the presence of high salt concentrations can influence spore surface
430 hydrophobicity (Wiencek et al., 1990; Isticato et al., 2019). This could effectively immobilize
431 spores onto surfaces within the repository, limit their migration, and mitigate actinide transport.

432 It is also important to note that these experiments were carried out in a pH range designed
433 for optimum Nd solubility and speciation and in simplified NaCl solutions. However, the pH
434 range expected for WIPP is much higher (~8-9), and WIPP brines contain other constituents that
435 can interact with Nd (e.g., sulfate, borate) or spore surfaces (e.g., Mg^{2+}, Ca^{2+}). This could affect

not only Nd speciation and solubility but also the availability of deprotonated binding sites on spore surfaces and the possible release and subsequent solubility of DPA. These experiments provide the basis for understanding future work that will focus on WIPP-specific conditions.

Studies of actinide bioassociation using halophilic organisms isolated from the WIPP have shown varying degrees of biological influence on actinide solubility and transport potential, including surface sorption, biomineralization, and biologically-induced precipitation (Francis et al., 1998; Ams et al., 2013; Bader et al., 2018). The results of this current study have shown that non-halophilic *Bacilli*, likely present at WIPP in spore form, can also influence the fate and transport of +3 actinides through biosorption or by generating solubility-enhancing complexants, such as DPA. The mechanism of influence appears to be dependent on biomass concentration as much as salt concentration. Whether these results also apply to other oxidation states remains to be tested.

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454

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456

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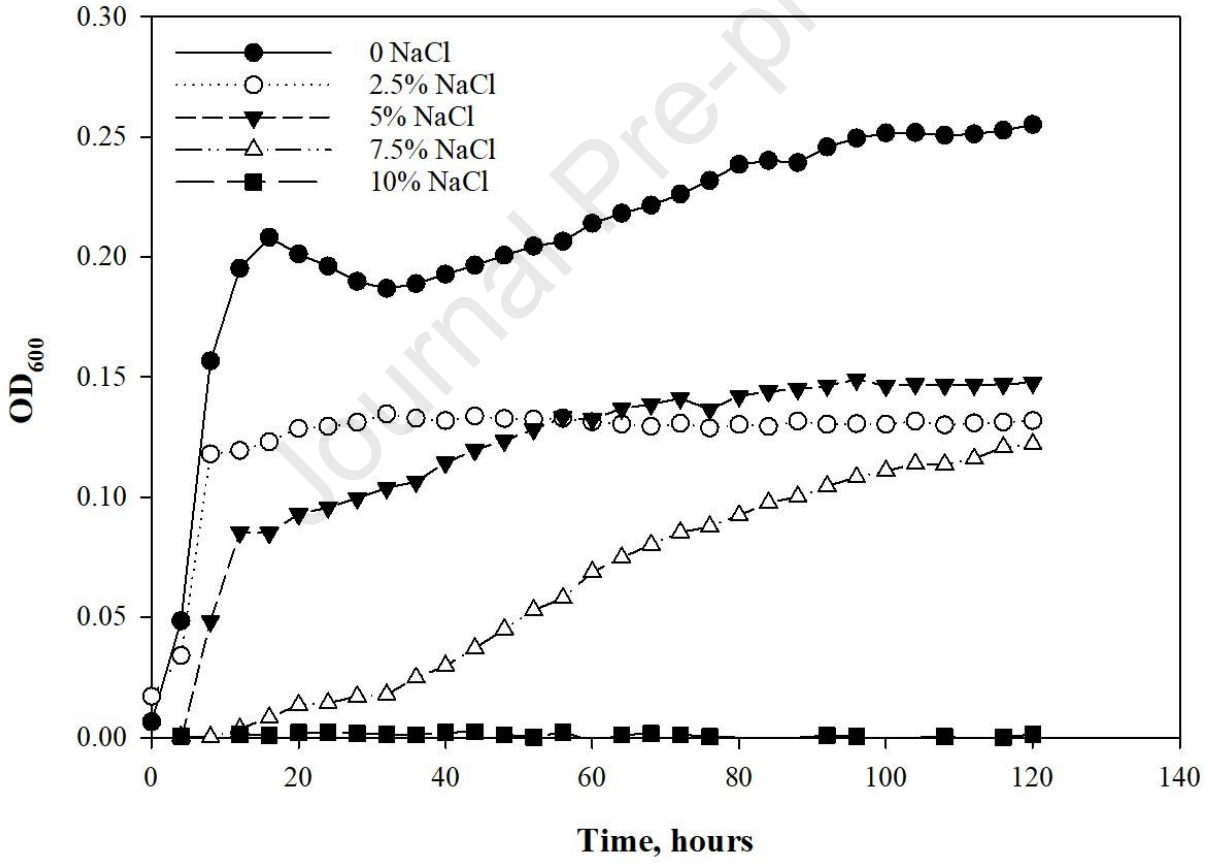
598 **SUPPLEMENTALS**

599

600 ***Bacillus* sp. WW-6-2 salt tolerance assay**. *Bacillus* sp. WW-6-2 was grown in R2B with added
601 NaCl concentrations ranging from 0 to 27.5% w/v (0-4.7 M) in increments of 2.5%. Cultures
602 were incubated at 28°C on an orbital shaker, using the Promega GloMax automated plate-reader
603 (Promega Corporation; Sunnyvale, CA). Growth was measured as the optical density at 600 nm.

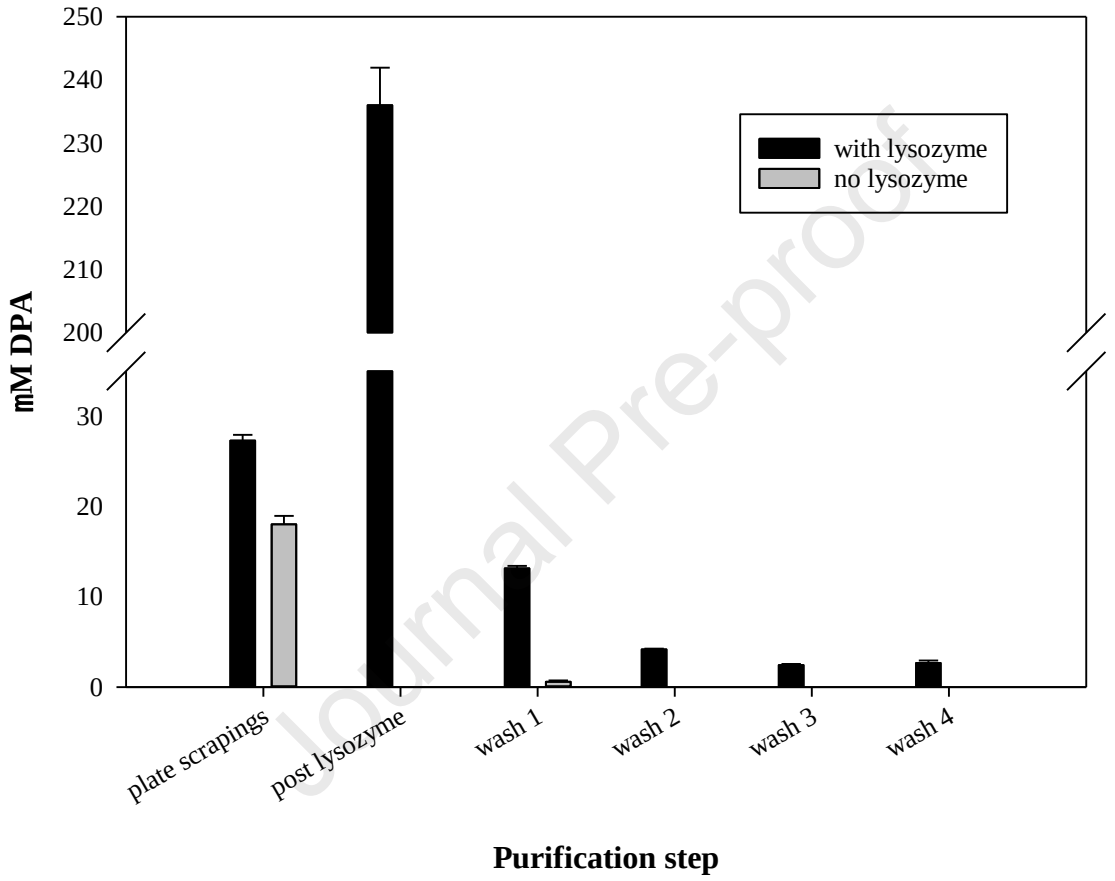


604

605 Figure S1. Growth of *Bacillus* sp. WW-6-2 in R2B with added varying concentrations of NaCl.

606

607 **DPA tracking during spore purification with and without lysozyme**. Samples were
608 withdrawn periodically during the spore purification procedures with and without lysozyme and
609 were measured with IC. No DPA was detected during the purification that did not use lysozyme.

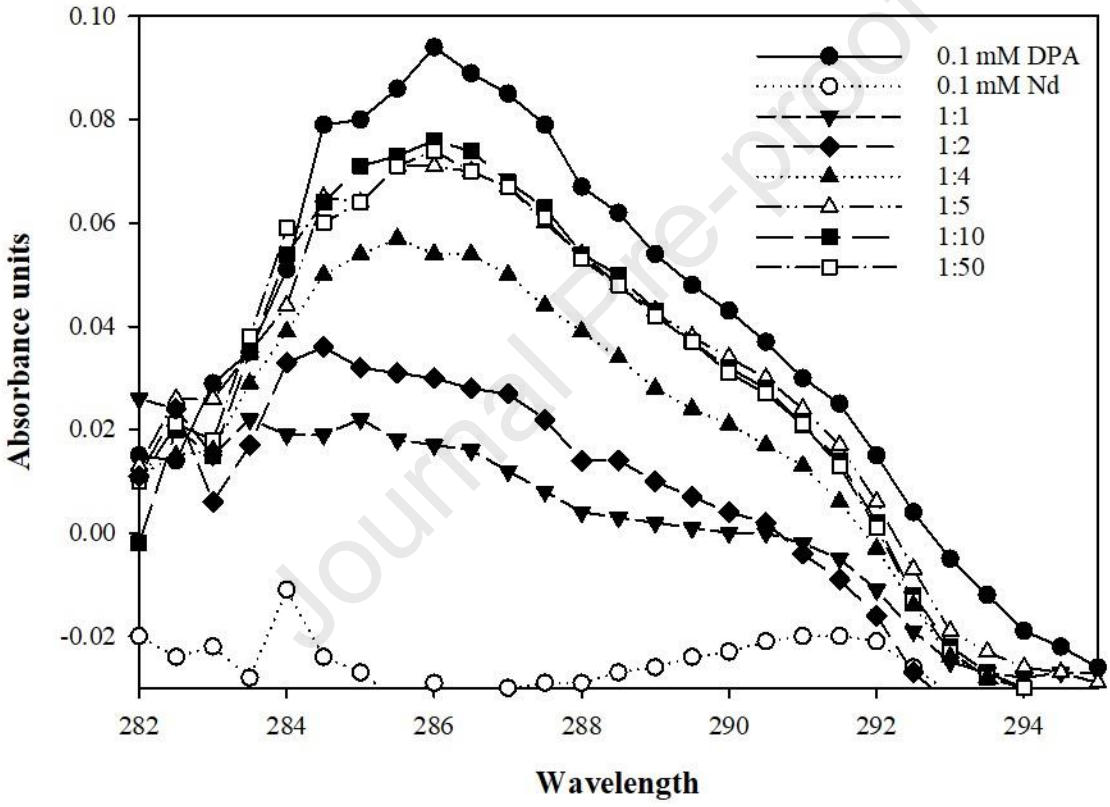


610

611 Figure S2. DPA release during spore purification procedure, with and without lysozyme step.

612

613 **Measurements of DPA and Nd-DPA complexes by UV-Vis spectrophotometry**. DPA was
614 measured on undiluted samples using the GeneSys 50 UV-Vis spectrophotometer (ThermoFisher
615 Scientific). Nd-DPA complexes were prepared in 25 mM nitric acid at different ratios: 1:1, 1:2,
616 1:4, 1:5, 1:10, 1:20, 1:50, and 1:100 with a constant concentration of 0.1 mM DPA. Samples
617 were scanned from ~280-310 nm (Lewis JC, 1967).



618

619 Figure S3. DPA spectra of Nd-DPA complexes at various ratios.

620

621

HIGHLIGHTS

- Spores of *Bacillus* species isolated from radioactive waste adsorb neodymium (Nd; analog of +3 actinide oxidation state)
- Sorption occurs only at high biomass concentrations but across low and high salt concentrations
- Spores release dipicolinic acid (DPA) which solubilizes Nd, even if adsorbed
- More DPA is released at higher biomass concentrations and lower salt concentrations
- Mechanisms of DPA release at low and high salt concentrations appear to be different
- DPA release occurs more quickly when lysozyme is used, but still occurs when lysozyme is omitted

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.