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LOS ALAMOS NATIONAL LABORATORY
CARLSBAD OPERATIONS

LCO-ACP-32, Revision 1

Biologically Influenced Transport of the +3 Actinide Oxidation State from the WIPP Environment, Using Neodymium as an Analog

Effective Date: 11/6/24

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0	12/15/22	All	Original Release
1	11/6/24	All	Updated to include new experimental data. Added other editorial changes as needed.

ACRONYMS AND ABBREVIATIONS

ACRSP	Actinide Chemistry and Repository Science Program
CFU	colony-forming unit
CRA	Compliance Recertification Application
DNA	deoxyribonucleic acid
DPA	dipicolinic acid (dipicolinate; pyridine-2,6-dicarboxylic acid)
EPS	extracellular polymeric substance
ERDA	Energy Research and Development Administration Well 6
GWB	Generic Weep Brine
ICP-MS	inductively-coupled plasma mass spectrometry
L	liter
M	molar; moles/liter
Mg	magnesium
ml	milliliter
mM	millimolar
µM	micromolar; micromoles/liter
Nd	neodymium
OD	optical density
PA	performance assessment
pC_{H+}	negative log of the hydrogen ion concentration
pH	negative log of the hydrogen ion activity
sp.	species (singular)
spp.	species (plural)
WIPP	Waste Isolation Pilot Plant
w/v	weight-to-volume

EXECUTIVE SUMMARY

This report provides an update to a previously issued report on the bioassociation of the +3 actinide analog, neodymium, with Waste Isolation Pilot Plant (WIPP) microbial isolates (LCO-ACP-32, *Biologically Influenced Transport of the +3 Actinide Oxidation State from the WIPP Environment, Using Neodymium as an Analog*). Results contribute to updates for the biocolloid parameters for the 2026 Compliance Recertification Application (CRA-2026).

At the time that LCO-ACP-32, Revision 0, was written, seven isolates had been tested in simplified NaCl solution, but only three (*Halobacterium* sp., archaeal isolate PB-1a, and *Chromohalobacter* sp.) had been tested in Generic Weep Brine (GWB) and Energy Research and Development Administration Well 6 (ERDA-6) brines. This report covers experiments on the remaining four isolates (archaeal isolate Trans-1b, *Salinicoccus* sp., *Nesterenkonia* sp., *Thalassobacillus* sp.) with neodymium (Nd) in WIPP brines.

The results of these experiments differ only slightly from those presented in the previous report, and the overall conclusions remain the same. In summary, all seven tested organisms influenced Nd concentrations in simplified NaCl and ERDA, but the time frame for loss from ERDA was slower than from NaCl. Previously, little to no biological influence was measured in GWB. This was determined to be due to competition between neodymium and magnesium (Mg) for surface complexation on the tested organisms. In the new experiments reported herein, biological influence was also low in GWB with the least influence among the most halophilic organisms. Similar behavior in NaCl and ERDA is likely due to the high concentration of NaCl in ERDA.

With additional testing, it also appears that organism behavior differs. Two of the tested organisms (*Salinicoccus* and *Nesterenkonia* spp.) formed large clumps immediately upon exposure to GWB, and cells settled to the bottom of the tubes. This settling behavior was observed in all tested organisms by the end of each experiment. Cells that are no longer in suspension are considered immobile and not vectors for actinide transport. In addition, spores of one tested organism (*Thalassobacillus* sp.) released a small amount of dipicolinate into solution which may have led to Nd resolubilization.

Overall, these data support the conclusion that matrix is a controlling factor on the influence of microorganisms on Nd in solution and that studies in WIPP-relevant matrices should be used to calculate biocolloid parameter values.

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1.0 INTRODUCTION

Potential biocolloid transport of actinides is an ongoing area of research for the Waste Isolation Pilot Plant's Compliance Recertification Application (WIPP CRA). This report provides an update on this work and supports the colloid enhancement parameters to be put forth in the parameter report for the CRA-2026 WIPP Performance Assessment (PA). The experiments described herein are only for the +3 actinide oxidation state.

Microorganisms can have varied influence on actinides in solution. In terms of performance assessment, "biocolloid" formation assumes mobilization and potential release. The term "bioassociation" is used in this report to encompass certain actinide-microbial interactions including surface complexation (and potential surface mineralization) and internal uptake. The term "biological influence", while inclusive of association phenomena, can also include induced precipitation, solubilization with microbially-generated complexants, or even redox transformations. This work is not focused on redox issues, but the generation of complexants is included where relevant.

To date, three Archaea and five Bacteria have been isolated from aerobic cultures of WIPP halite and/or brine seep stalactites at NaCl concentrations ranging from 10-27.5% (1.7-4.7 M; Swanson et al., 2012; Swanson and Reed, 2018).

Table 1. Organisms isolated from WIPP halite

Organism	Domain/Phylum	Optimum [NaCl] for Growth, M (range)
Halobacterium sp. (*noricense*)	Archaea/Euryarchaeota	3.42 (1.71-saturated)
Isolate PB-1a		4.28 (2.14-saturated)
Isolate Trans-1b		2.99 (1.71-saturated)
Chromohalobacter sp. (*salexigens*)	Bacteria/Proteobacteria	2.57 (0.86-4.28)
Salinicoccus sp. (*roseus*)	Bacteria/Firmicutes	1.71 (0.43-2.99)
Nesterenkonia sp.	Bacteria/Actinobacteria	0.43 (0-2.57)
Thalassobacillus sp. (*cyri*)	Bacteria/Firmicutes, spore forming organisms	0.76 (0.43-2.14)
Bacillus sp.		0.33 (0.33-0.86)

One of the archaea, *Halobacterium* sp., has been used as a representative organism for study in Actinide Chemistry and Repository Science Program (ACRSP) experiments, since it is also one of the most commonly found haloarchaea in subterranean salts (Jaakkola et al., 2016). It has been tested for its ability to associate with uranium, neodymium, and cadmium (Showalter et al., 2016; Bader et al., 2017, 2019; Swanson, 2019; Liu et al., 2020). Neptunium(V) biosorption was tested with the halophilic bacterium, *Chromohalobacter* sp., and some preliminary data also exist for this organism with thorium (Ams et al., 2013; Reed, 2013). Neodymium association experiments were carried out on the majority of organisms listed above and on spores of a *Bacillus* sp. that was isolated from WIPP-bound waste (Swanson et al., 2021; Swanson et al., 2023).

From the above list, only the three archaea and *Chromohalobacter* sp. are able to grow in WIPP brines amended with substrates and nutrients. In comparison, all are able to survive exposure to GWB and ERDA brines for a period of weeks (testing ranged from 3-39 weeks) at high biomass concentrations before resuscitation becomes impossible. At lower biomass concentrations, survival is shorter, but these timeframes are inconsequential when compared to the 10,000-year repository lifetime. Those that form spores survive much longer and could remain throughout this timespan.

This report presents data on the behavior of Nd with the remaining WIPP isolates in anoxic WIPP brines. Neodymium was used as an analog for the +3 actinide oxidation state. Simplified NaCl was used to screen the potential for biological influence at each organism's optimum salt concentration, since published data on biosorption are most often generated in simple matrices. Experiments were then extended to anoxic WIPP brines, whose chemistry and pH can significantly affect outcome. These results are compared to previous data in LCO-ACP-32, Revision 0, and the overall conclusions remain the same: the background matrix affects biological influence, and data generated in WIPP brines should be used to calculate parameter values.

2.0 SUMMARY OF EXPERIMENTS

All organisms were grown in their optimal media at their optimal NaCl concentration (usually referred to by weight/volume percentage, or w/v). At early stationary phase, cells were harvested and washed five times with solution containing optimal NaCl, and the cell pellet was stored overnight at 4°C. For the initial experiments, pellets were resuspended in simplified NaCl solutions at each organism's optimum. For experiments in WIPP brines, pellets were brought into a nitrogen-filled glove box and resuspended in anoxically prepared, 90% formulations of GWB and ERDA-6 brines. All experiments targeted an optical density (OD) equivalent to ~10^9 cells/ml. Optical density values and cell numbers were correlated prior to the start of each bioassociation experiment.

Concentrated neodymium stock solutions (~ 2 mM) were prepared by adding solid $NdCl_3$ to each salt solution (solutions ranged between 15-30% NaCl). Working solutions containing twice the target Nd concentration (2X) were prepared from these stocks in each respective solution and in GWB and ERDA brines (target 2X Nd concentrations in WIPP brines ≈ 0.4 µM; target Nd in salt solutions was higher, since solubility was not an issue). The cell suspension was then mixed 1:1 with the 2X Nd-NaCl working solution. Cell suspensions were placed on a rotator at ambient temperature (simplified NaCl experiments in ambient atmosphere, WIPP brine experiments in anoxic atmosphere), and samples were withdrawn periodically for analysis by inductively-coupled plasma-mass spectrometry (ICP-MS) and ion chromatography and for measurements of OD, microscopic counts or plate counts.

Optical density readings were taken at each sampling point, after which samples were passed through an Amicon 100 kDalton centrifugal filter and then diluted in 2% nitric acid. Measurements of Nd in the filtrate are considered to be "dissolved" Nd. The filtration step

captures surface sorption, internal uptake, and induced precipitation and/or mineralization either at the cell's surface or from the bulk solution. Abiotic controls were run parallel to the biotic samples to account for any precipitation not caused by the presence of organisms. This was especially important in ERDA where Nd concentrations in abiotic samples often decreased with time. Pooled filtrates were used to measure pH and for analyzing dipicolinate (DPA) concentrations, where relevant. Direct microscopic counts or colony-forming unit (CFU) counts were also obtained, when possible.

The percentage of biologically-influenced Nd was calculated as:

$$\{[(Nd\ in\ abiotic\ sample) - (Nd\ in\ biotic\ sample)]/(Nd\ in\ abiotic\ sample)\} * 100$$

3.0 RESULTS

To date, seven WIPP microbial isolates have been tested for bioassociation with Nd in both simplified NaCl solution and in WIPP brines. These organisms include: archaeal isolate PB-1a, *Halobacterium* sp., archaeal isolate Trans-1b, *Chromohalobacter* sp., *Salinicoccus* sp., *Nesterenkonia* sp., and spores of *Thalassobacillus* sp. and *Bacillus* sp. isolated from WIPP-bound waste. Results for *Halobacterium* sp. and the waste *Bacillus* sp. have been presented elsewhere (Swanson, 2019; Swanson et al., 2021) but will be reviewed here for comparison.

Throughout this section, changes in Nd concentration will be referred to as "loss" or "removal from solution." This loss is reflected as an increase in the "percent of biological influence." Summary graphs presenting these data for each tested organism are presented in the Appendix.

3.1 Bacteria

3.1.1 *Chromohalobacter* sp. (putatively *salexigens*)

This organism is a member of the Gamma Proteobacteria. It is a Gram-negative, borderline extreme halophile, capable of slow growth in WIPP brines but growing optimally at 2.57 M NaCl. Members of this genus have been found to produce extracellular polymeric substances (EPS) that may contribute to metal or actinide surface complexation (Biswas and Paul, 2014). This isolate has been tested previously for its ability to adsorb neptunium in perchlorate solutions (Ams et al., 2013) and neodymium in WIPP brine.

Ninety-seven percent of the Nd in NaCl solution with *Chromohalobacter* sp. was immediately removed and remained so throughout the time course of the experiment (Figures A1 and A2). Abiotic levels of Nd in solution also decreased slightly.

In WIPP brines, there was approximately 40% loss of added Nd from biotic GWB samples and little to no loss from abiotic samples throughout the course of the experiment (Figures A3 and A4). In contrast, loss from the ERDA biotic samples was immediate and significant and reached near 100% at approximately 5 weeks (Figures A5 and A6). There was also ~40% decrease in Nd in the ERDA abiotic samples by 5 weeks.

There was no significant change in cell numbers in the simple NaCl experiments. In WIPP brines, there was a gradual decrease in colony-forming unit counts in both brines within the first week, and a significant decrease by week 2. At the final time point, there were no viable cells in the ERDA samples and ~10^6 CFU/ml in GWB (Figure 1).

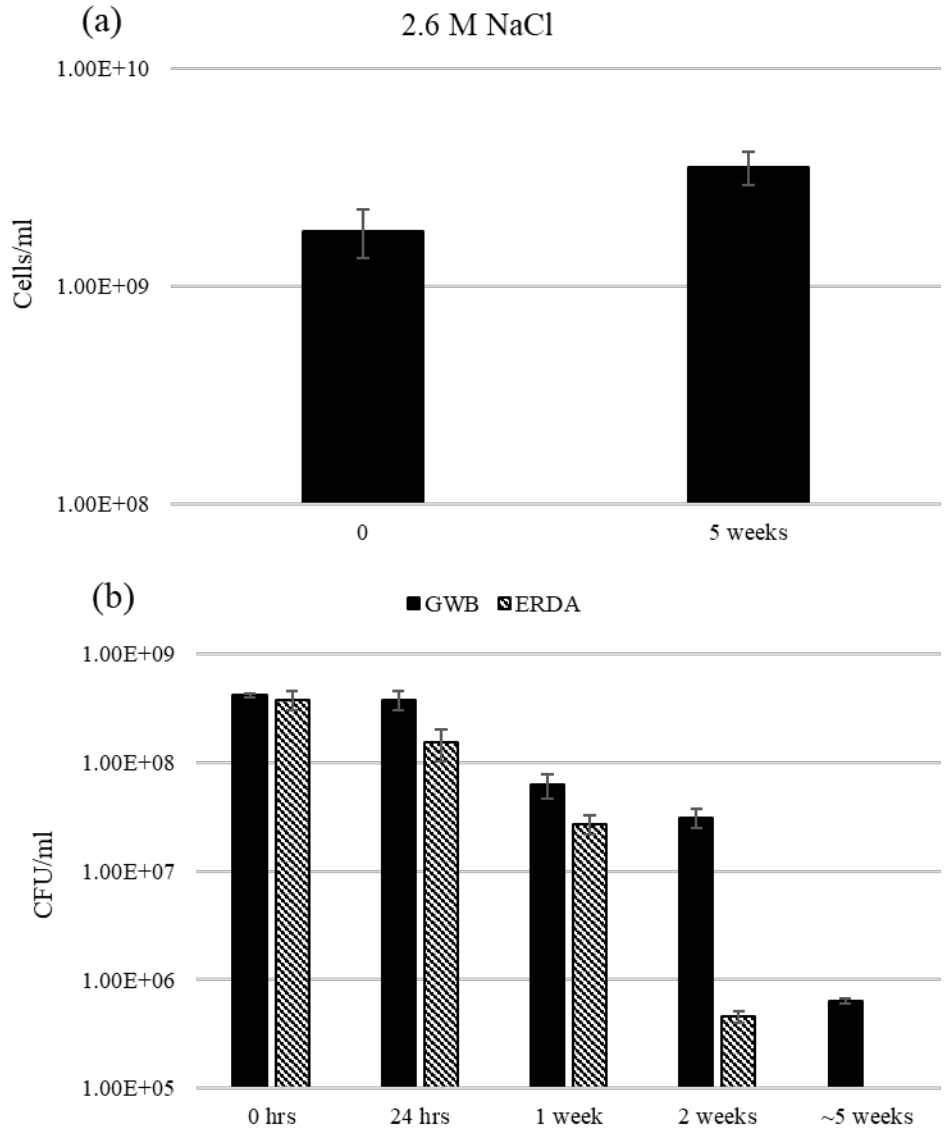


Figure 1. *Chromohalobacter* sp. CFU counts over time in a) NaCl and b) GWB and ERDA experiments

3.1.2 *Salinicoccus* sp. (putatively *roseus*)

This organism is a member of the Phylum Firmicutes. It is a moderately halotolerant, pink-pigmented, Gram-positive coccus and obligate aerobe. Members of this genus produce EPS and form clumps when grown in broth that are often difficult to measure by OD or microscopic counts. By microscopy, it often appears in tetrads or larger aggregates of up to 12 cells. This clumping is significant in that it can affect the surface area of cells exposed to brine and Nd. *Salinicoccus* sp. cannot grow in WIPP brines, but it can be resuscitated after exposures of 23-25 weeks in GWB and ERDA, respectively, but only when tested at high biomass concentrations (~1E+09 cells/ml).

Neodymium biosorption onto *Salinicoccus* sp. was immediate and measured approximately 39%. It increased to 55% over a 24-hour period and leveled off at 60% by one week (Figure A7). However, at one month, apparent bioassociation was 97% (Figure A8). Neodymium was also lost from the abiotic samples over time.

Experiments with *Salinicoccus* sp. in WIPP brines were conducted twice. In the first round, cells immediately clumped together in GWB and sedimented to the bottom of each tube (Figure 2). This prevented cell counting and may have affected Nd in solution. By 24 hours, all cells had aggregated in ERDA as well. The results presented here are from the second round of experiments.

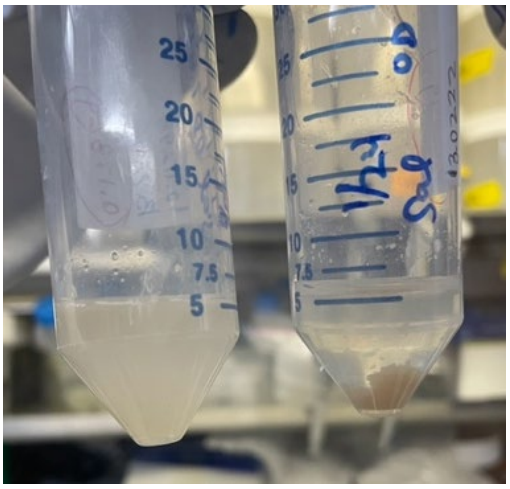


Figure 2. *Salinicoccus* sp. suspensions in WIPP brines from the first round of experiments: ERDA left, GWB right

In GWB, the loss of Nd from solution within the first week was negligible (<20%) over the first 24 hours and apparent bioassociation only reached ~40% at the end of the experiments (Figures A9 and A10). In ERDA, biological influence was immediate (60% at t = 0) and near-complete Nd removal (96%) was observed within the first 12 hours (Figures A11 and A12).

Cell counts in NaCl experiments did not vary significantly over time (Figure 3a). In contrast, CFU counts decreased over an order of magnitude in GWB and two orders of magnitude in ERDA over time (Figure 3b).

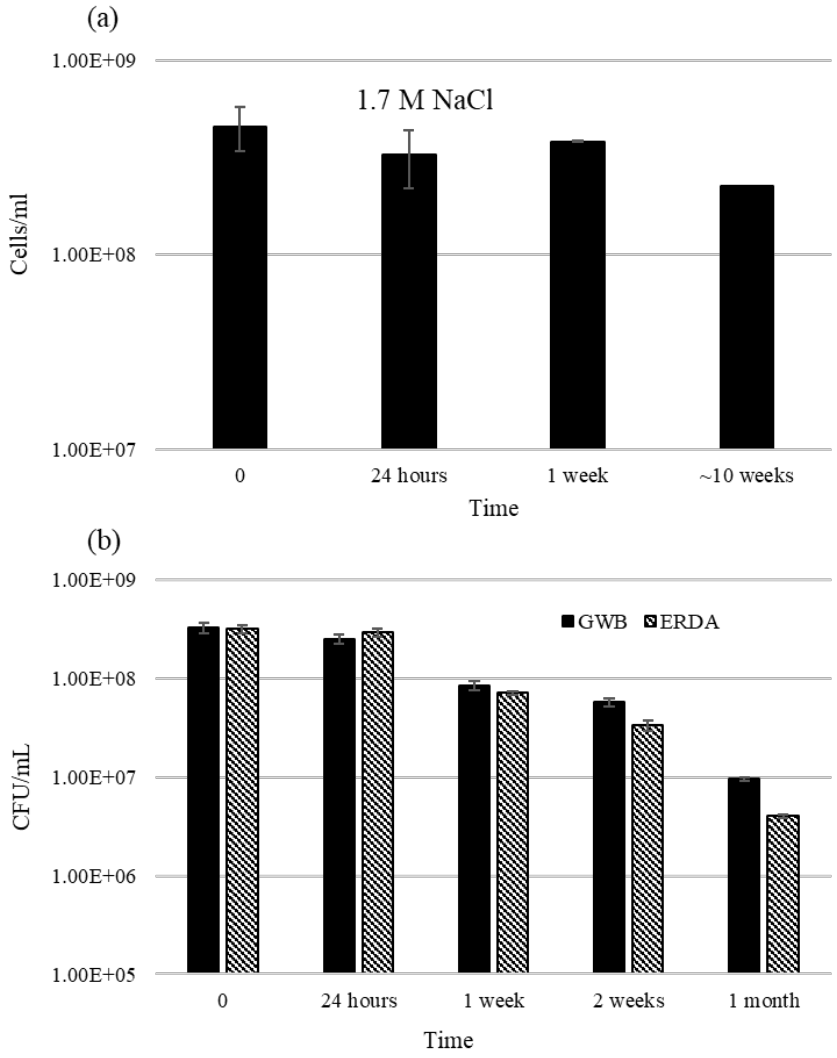


Figure 3. Cell counts of *Salinicoccus* sp. in (a) NaCl and (b) WIPP brines over time

3.1.3 *Nesterenkonia* sp.

Nesterenkonia sp. is a member of the Phylum Actinobacteria. It was isolated from two different samples of WIPP halite (near-pure halite and argillaceous halite) both incubated in generic halophile broth containing 10% NaCl. It is Gram-positive and halotolerant with an optimum NaCl concentration for growth of ~2% (~0.3 M), but it is capable of growth between 0-17% NaCl (~2.9 M). Members of the Actinobacteria are notable for their resistance to environmental stressors, such as pH and temperature extremes, heavy metals, solvents, ionizing radiation, and desiccation.

Neodymium removal from 0.43 M NaCl solution by *Nesterenkonia* sp. was immediate and near complete, with over 99% loss from solution throughout the course of the experiment (Figures A13 and A14). *Nesterenkonia* spp. cells, like *Salinicoccus* spp., often appear as couplets or tetrads.

In GWB, Nd loss from solution was slow, beginning after 4 days, and apparent association reached a maximum of only 50% at one month (Figures A15 and A16). In ERDA, loss was gradual and near complete by one week, with a maximum apparent association of 96% (Figures A17 and A18).

In all experiments, regardless of matrix, morphology remained coccoid. Like many Actinobacteria, *Nesterenkonia* spp. can alter morphology (and hence, surface area) from cocci to short rods during their log growth phase. Cell counts did not change significantly over the course of the experiment in NaCl, but CFU counts dropped over an order of magnitude in GWB and approximately two orders of magnitude in ERDA at one month (Figure 4).

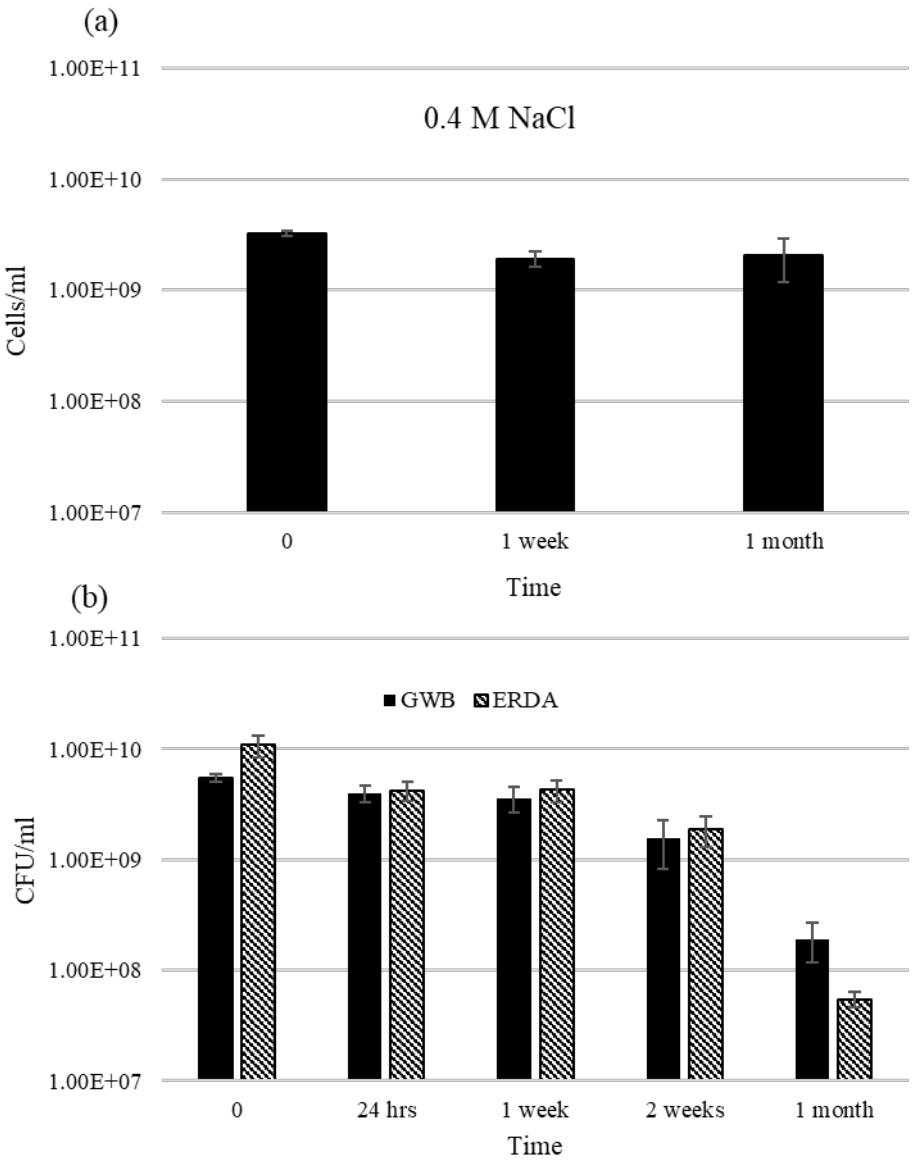


Figure 4. Cell counts and CFU counts of *Nesterenkonia* sp. over time in (a) NaCl and (b) WIPP brines

3.1.4 *Thalassobacillus* sp. spores

Thalassobacillus sp. was isolated from a stalactite sample formed from brine seep and incubated in growth medium containing 10% NaCl (1.71 M). It is a Gram-positive, slightly halophilic spore forming bacillus and does not grow in the absence of NaCl. In its spore form, it can likely survive for long periods in WIPP brines (longest tested was 39 weeks). Prior to beginning these experiments, spores were purified with and without lysozyme and all wash solutions were tested for the presence of dipicolinate (DPA), a ligand produced and released by some spores that can

enhance actinide solubility. These spores were not too susceptible to lysozyme treatment, but since some DPA was detected (~8 μM), lysozyme was omitted from the procedure.

Thalassobacillus sp. spore influence on Nd in 0.76 M NaCl solution was immediate, with approximately 78% loss from solution at time zero (Figures A19 and A20). This increased slowly over time until 98% of Nd was lost by one month. This is in contrast to previous experiments with spores from a different organism, where the release of DPA caused all Nd to return to solution (Swanson et al., 2021). Samples taken from the original *Thalassobacillus* sp. spore suspension that had been stored for the duration of these kinetics experiments had approximately 0.17 μM DPA, which might not have been enough to cause solubilization in these samples.

Removal of Nd by spores in GWB was gradual and reached a maximum of 76% at 2 weeks (Figures A21 and A22). At one month, a small amount of Nd had returned to solution. In ERDA, Nd removal was immediate at ~80%, but over time this decreased to ~ 30% at one month (Figures A23 and A24). Concentrations of Nd in the abiotic ERDA samples also decreased significantly.

DPA was released in both GWB and ERDA brines immediately upon exposure to brine. Measurements decreased over time in both brines, suggesting no subsequent release after the initial, but levels dropped more quickly in GWB than in ERDA (within first 12 hours in GWB and at 72 hours in ERDA; Figure 5).

Colony-forming unit counts remained stable over time in GWB, but decreased by an order of magnitude in both NaCl and ERDA matrices (Figure 6).

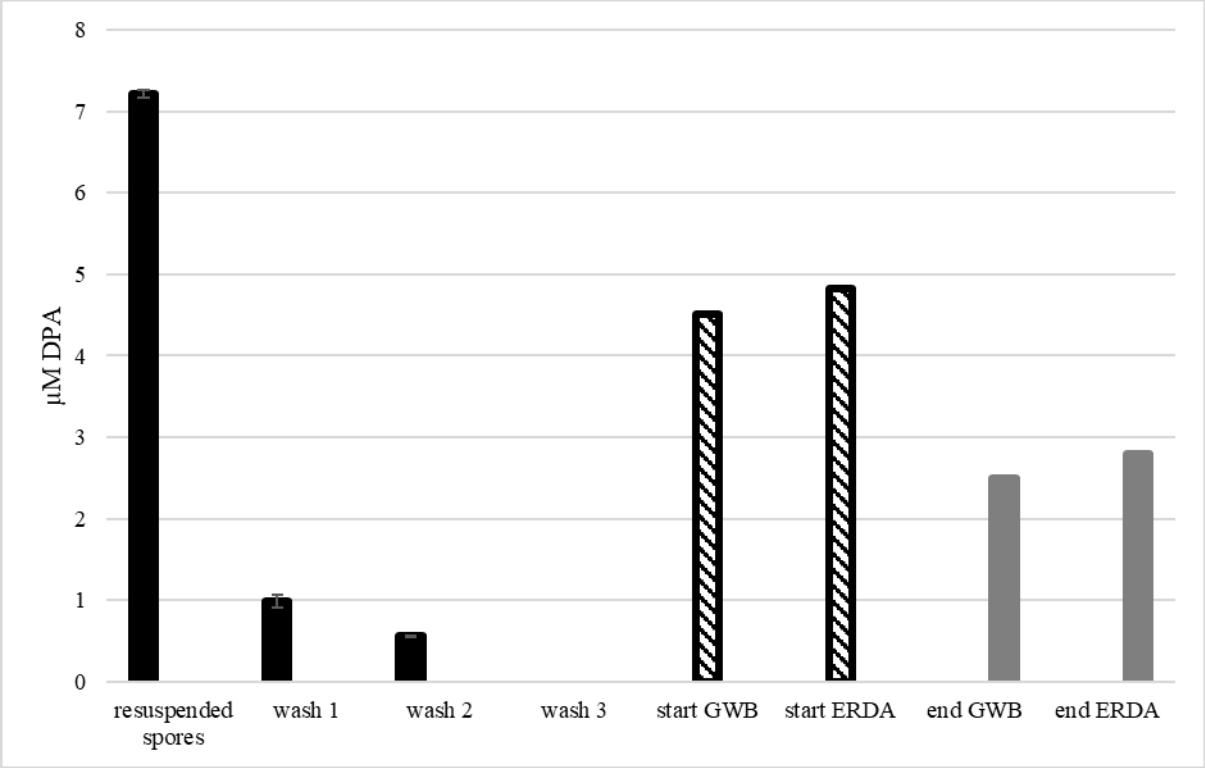


Figure 5. DPA measured during spore purification (black), at the start of experiments (diagonal) and at the end of experiments (gray). A total of 5 washes were performed before beginning the experiment. No DPA was measured in washes 4 and 5, so these are not shown. Note also that measurements in brines were not made in replicate, due to limited sample volume and the need to pool the filtrates for pH measurements as well.

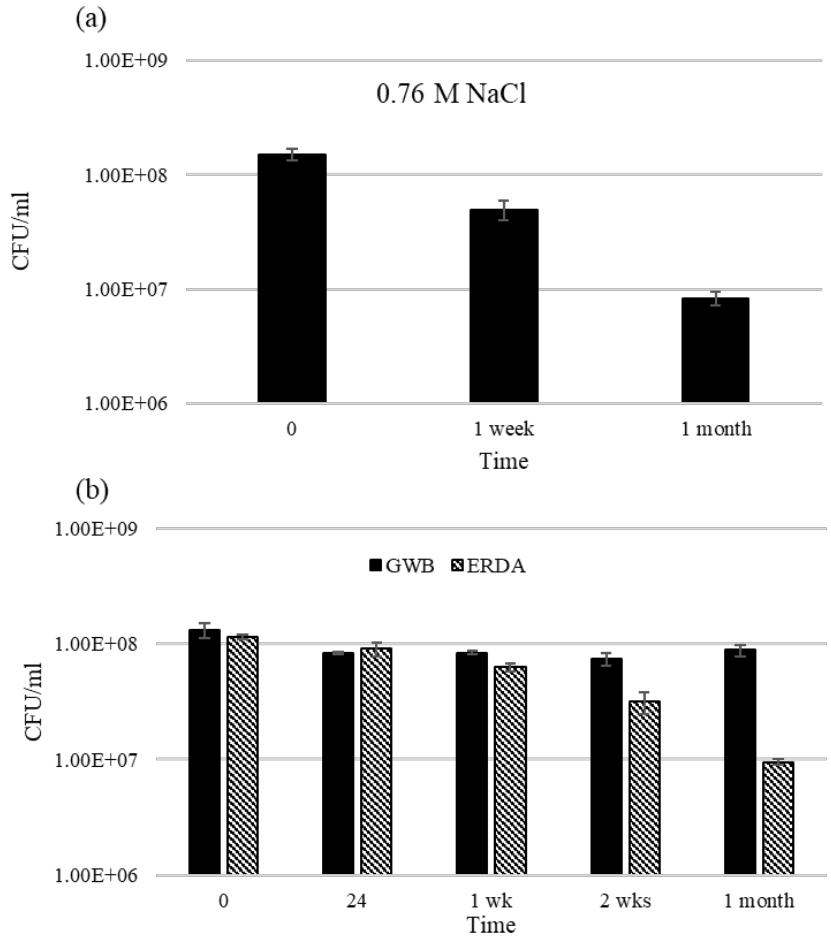


Figure 6. CFU counts of *Thalassobacillus* sp. in (a) NaCl and (b) WIPP brines over time

3.2 Archaea

3.2.1 PB-1a

Archaeon PB-1a is the most halophilic of the WIPP archaeal isolates, with an optimum NaCl concentration for growth of 25% (4.28 M). It has not yet been identified taxonomically, but it was verified as an archaeon by amplification of its 16S ribosomal DNA using archaeal primers.

Neodymium loss from 4.28 M NaCl solution was gradual and took over 48 hours before it was completely removed from solution. Approximately 10% of Nd was lost from the abiotic samples over time (Figures A25 and A26).

There was little to no loss of Nd from GWB in either biotic or abiotic samples over time (Figures A27 and A28). In contrast, approximately 70% of Nd was lost from biotic ERDA samples by 48 hours, but its concentration did not change for the remainder of the experiment (Figures A29 and A30). Cell numbers remained constant throughout (Figure 7).

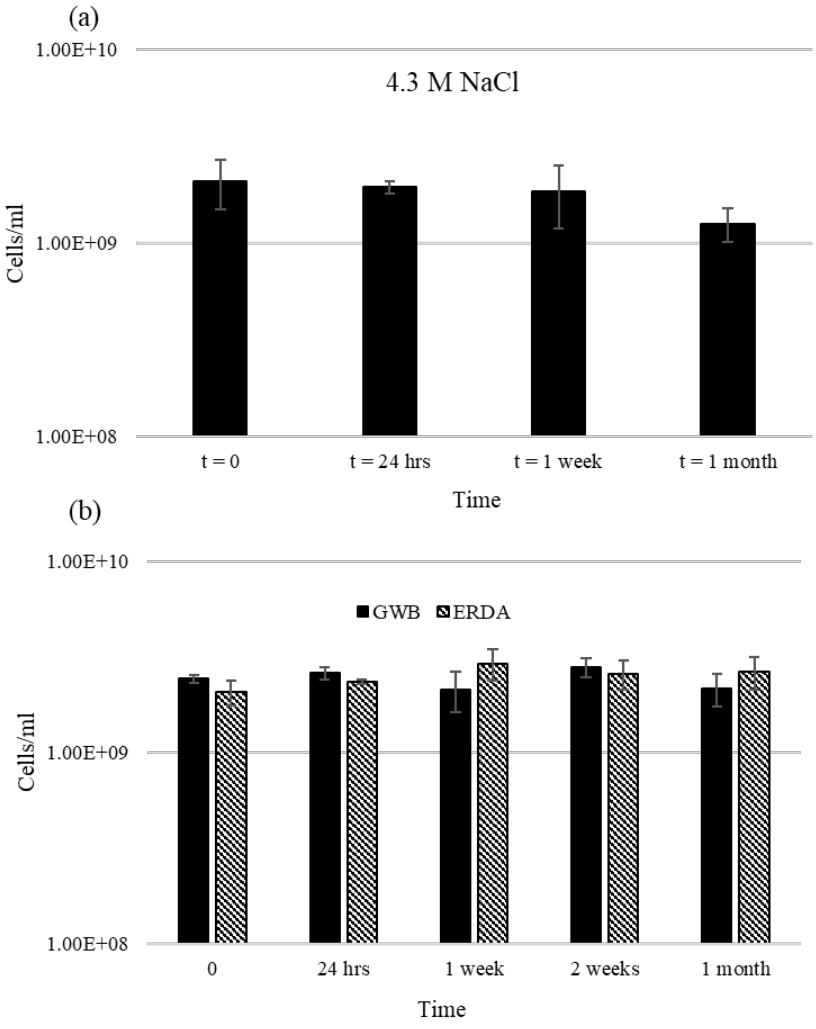


Figure 7. Cell counts for archaeal isolate PB-1a over time in (a) NaCl and (b) WIPP brines

3.2.2 Trans-1b

Archaeon Trans-1b is the least halophilic of the WIPP archaeal isolates, with an optimum NaCl concentration for growth of 17.5% (2.99 M). It has not yet been identified taxonomically, but it was verified as an archaeon by amplification of its 16S ribosomal DNA using archaeal primers.

Approximately 30% of Nd was removed from NaCl solution immediately upon exposure, 80% was removed by 24 hours, and all was removed by day 5 (Figures A31 and A32). In WIPP brines, there was little to no loss of Nd (<20%) from GWB in either biotic or abiotic samples over time (Figures A33 and A34). Neodymium was lost gradually from biotic ERDA samples, reaching ~84% apparent sorption by 24 hours and 97% by 2 weeks. Measured values in ERDA were low for both biotic and abiotic samples, but the percent of influence could still be validly calculated (Figures A35 and A36). Cell numbers were not tracked during this experiment, as the organism did not take up the nucleic acid stain sufficiently and CFU counts are difficult for

archaea due to the length of time required for growth and the difficulty in counting the pinpoint colonies that grow. However, if OD values are used as a proxy (which appears to be valid for haloarchaea), cell concentrations remained consistent over the time course of the experiments in GWB and decreased over time in ERDA by about half an order of magnitude (which is close to the approximate uncertainty for microscopic cell counts).

3.2.3 *Halobacterium* sp. (putatively *noricense*)

This organism was tested previously, and results of those experiments appear in report LCO-ACP-23, *Review of the Microbial Colloid Contribution to the WIPP Actinide Source Term*. To summarize, neodymium loss from 3.4 M NaCl solution was gradual but complete over the first 24 hours, but only at higher biomass concentrations (5.03E+09 cells/ml). By one month, approximately 50% of Nd was also lost from suspensions containing lower biomass concentrations (2.02E+08 cells/ml).

There was almost no influence of organisms on Nd solubility in GWB. In ERDA, the percent of influence increased slowly with time and reached 78% at 5 weeks.

4.0 DISCUSSION AND CONCLUSIONS

4.1 Comparison between organisms in each brine

Experiments were carried out with 7 prokaryotes—3 Archaea and 5 Bacteria (Figures 8-10). All Archaea were of the Phylum Euryarchaeota, Class Halobacteria. The Bacteria belonged to 3 different phyla: Proteobacteria (1 isolate), Actinobacteria (1 isolate), and Firmicutes (3 isolates). Of the Firmicutes, two were bacilli that were tested in spore form (one from previous experiments). Three organisms are Gram-negative, and four are Gram-positive. Four are pigmented, but their pigments are not the same. Extracellular polymeric substance production and the presence of a glycoprotein surface layer (S-layer) were not determined experimentally, but studies have found that two are known to produce EPS—*Salinicoccus* sp., *Chromohalobacter* sp.—and four are known to have S-layers—*Halobacterium* sp., isolate PB-1a, isolate Trans-1b, and *Bacillus* sp. (Biswas and Paul, 2014; Sidhu and Olsen, 1997). A summary of these surface characteristics is shown in Table 2.

Table 2. Test organism characteristics that can affect surface sorption

Organism	Gram Stain Reaction	S-Layer	EPS	Vegetative vs. Spore	Pigment	Test w/v % NaCl (optimum NaCl)
Halobacterium sp.	−	+	−	V	+	20
Isolate PB-1a	−	+	−	V	+	25
Isolate Trans-1b	−	+	−	V	+	17.5
Chromohalobacter sp.	−	−	+	V	−	15
Salinicoccus sp.	+	−	+	V	+	10
Nesterenkonia sp.	+	−	−	V	+	1.95
Thalassobacillus sp.	+	−	−	S	−	4.45
Waste *Bacillus* sp.	+	+	−	S	−	0.85

The only two findings of note, based on organism type, are for *Salinicoccus* sp. and the spores of *Bacillus* sp. and *Thalassobacillus* sp.: 1) cell clumping might have reduced the surface area available for sorption in the case of *Salinicoccus* sp. and 2) the release of DPA by spores led to resolubilization of Nd over time.

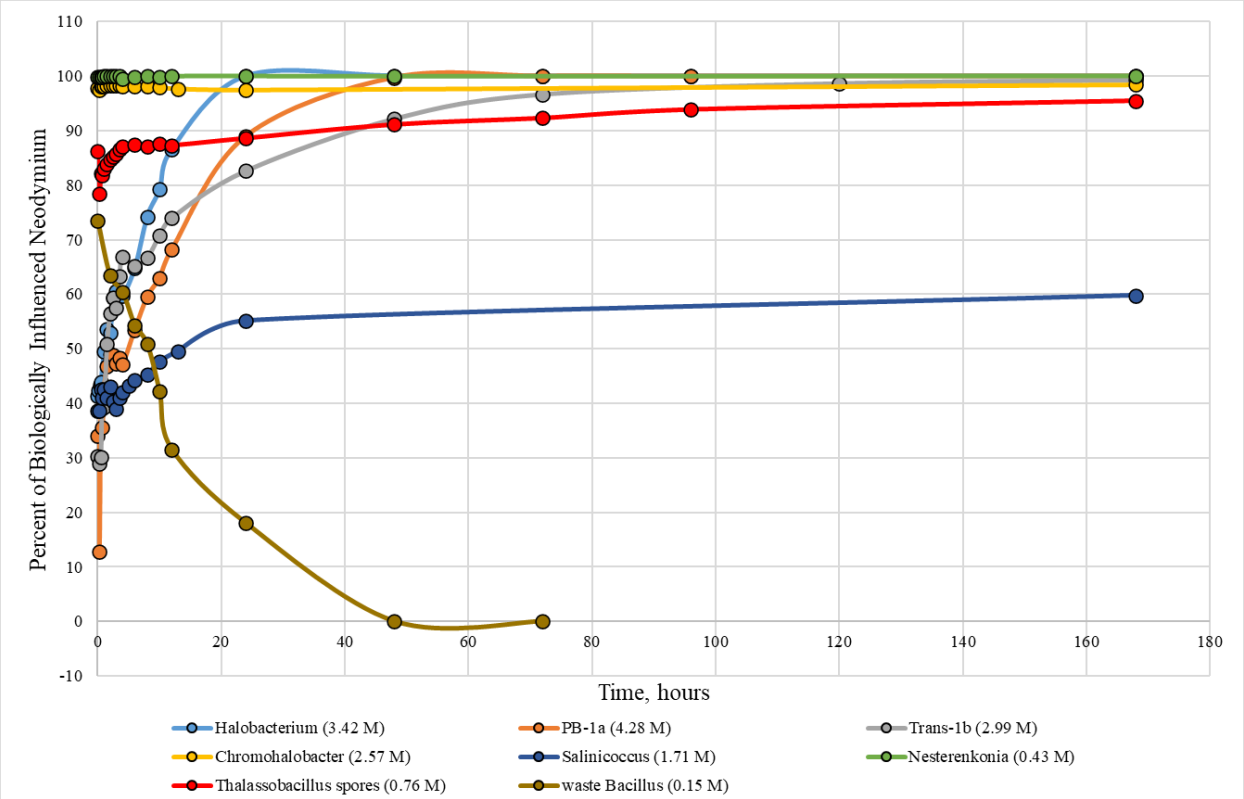


Figure 8. Comparison of all tested organisms in simple NaCl matrix at each organism's optimum, over the course of one week. *Halobacterium* sp. and *Bacillus* sp. have been presented elsewhere but are included here for comparison (Swanson, 2019; Swanson et al., 2021).

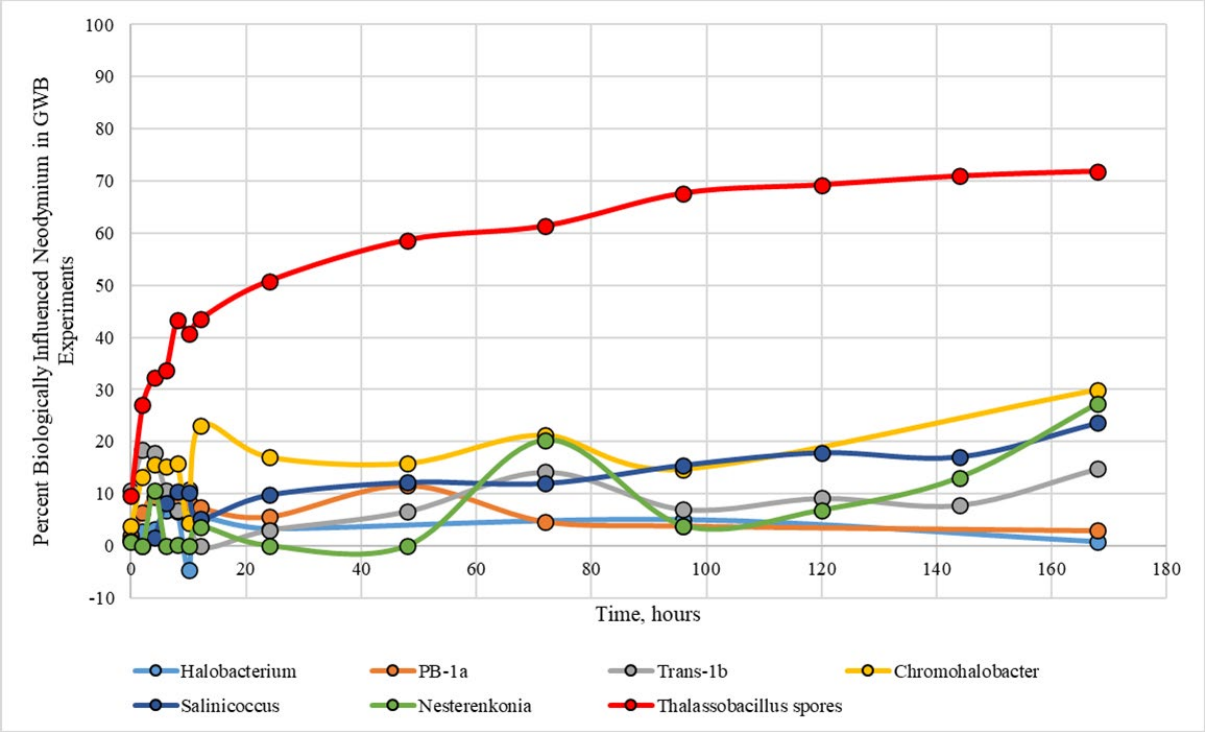


Figure 9. Comparison of all tested organisms in GWB over the course of one week

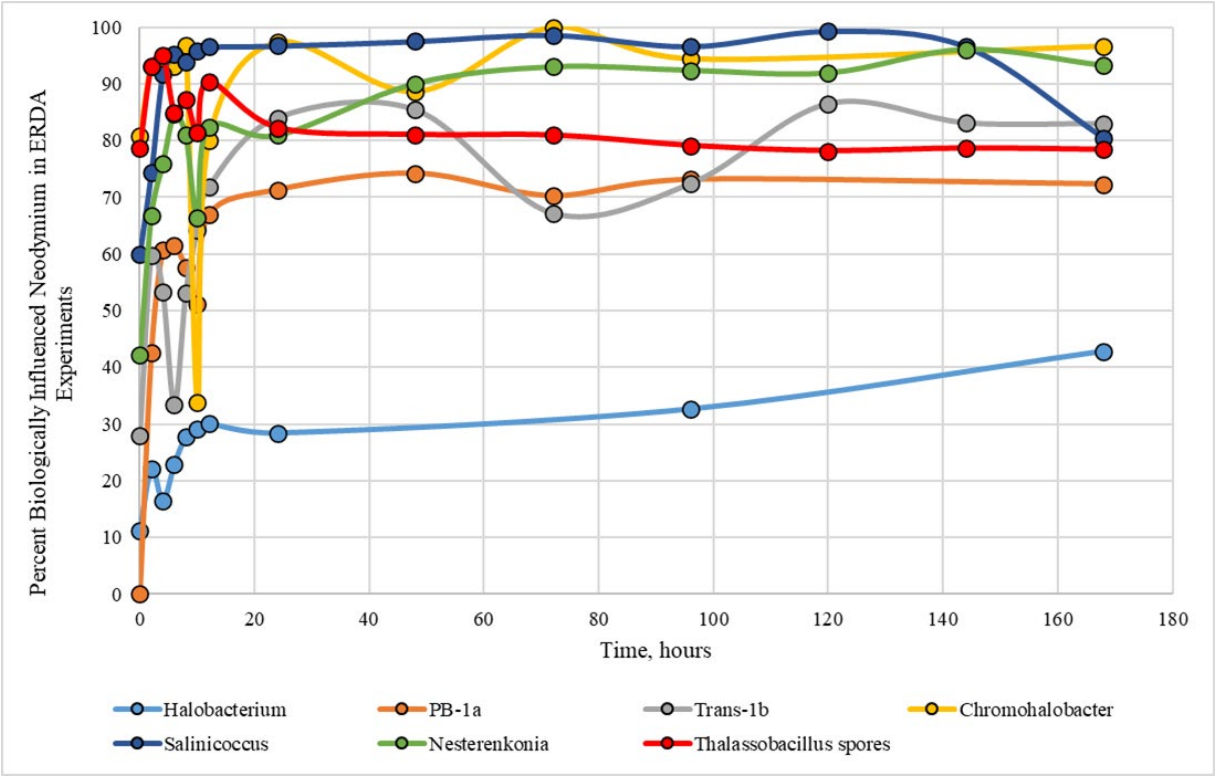


Figure 10. Comparison of all tested organisms in ERDA over the course of one week

4.2 Differences in biomass concentration

Not all organisms were tested for biomass dependence. For those that were (*Halobacterium* sp. and waste *Bacillus* sp.), a significant increase in Nd removal from solution was observed at higher biomass concentrations. This agrees with the literature and is logical based on the increased number of available surface sites with increasing biomass.

In all WIPP brine experiments with bacteria, viable cell numbers decreased over time, often significantly (see Figures 1b, 3b, 4b, and 6b). This is due to the higher ionic strength in the WIPP brines compared to each organism's optimum NaCl concentration and the inability of the bacteria to maintain osmotic balance in these conditions. Counts in ERDA were also lower than those in GWB, due to the much higher concentration of NaCl in that brine. This loss in numbers does not correspond to a decrease in apparent association in ERDA, which could suggest association with sedimented dead cells or cell debris or some other means of induced precipitation. Counts for archaea were not affected by the WIPP brines.

4.3 Differences between NaCl concentrations

A weak negative correlation can be seen between the initial percentage of biological influence and the test matrix NaCl concentration (Figure 11; note that background NaCl concentrations are based on each organism's optimum for growth). This type of correlation was also observed with *Chromohalobacter* sp. in neptunium-perchlorate solutions (Ams et al., 2013). However, this

correlation disappears completely over time, suggesting that in the long term, Nd disposition is controlled by a mechanism other than surface complexation.

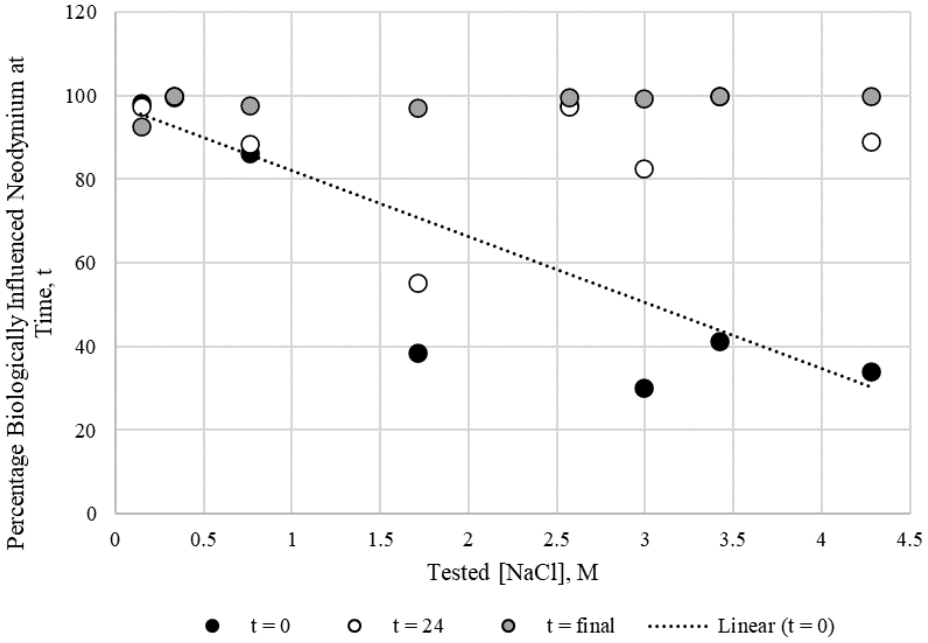


Figure 11. Correlation between the percent of biological influence and test [NaCl] at three timepoints. Each point within a series represents a different test organism.

4.4 Differences between simple NaCl and WIPP brines: matrix effects

In the original revision of this report, only three of the seven isolates had been tested in WIPP brines—*Halobacterium* sp., isolate PB-1a, and *Chromohalobacter* sp. Findings were similar for all three organisms. There was little influence of the organism on Nd concentration in GWB, but Nd was lost from solution in ERDA, especially when organisms were present.

The results for Trans-1b, *Salinicoccus* sp., and *Nesterenkonia* sp. also followed the same trend, but biological influence on Nd in GWB with *Thalassobacillus* sp. spores did not (Figure 12). The most halophilic organisms resulted in the least association in GWB (less than 20% in *Halobacterium* sp., PB-1a, and Trans-1b experiments). The most halophilic bacteria (*Chromohalobacter* sp. and *Salinicoccus* sp.) had moderate influence on Nd in GWB (<40%), but the effect of *Salinicoccus* sp. was only observed at the final time point. The reason for this late increase is unknown but could be due to precipitation of Nd with clumped and sedimented cells.

Results in ERDA and NaCl are more similar to each other than to GWB, likely because of the high concentration of NaCl in ERDA. For the most extreme halophiles (*Halobacterium* sp. and isolate PB-1a), the biological influence in ERDA is lower than in NaCl, while for most of the

other organisms, it is similar. The exception is *Salinicoccus* sp., and again, this could be due to the clumping effect.

Finally, *Thalassobacillus* spore behavior is different from the other organisms, as discussed in Section 4.1, and also because its spores are likely less affected by the high ionic strength of the brines as the vegetative bacterial cells. It is interesting to note the difference between spore influence by *Thalassobacillus* sp. and the waste *Bacillus* sp., due to the release of DPA (Figure 8).

The similarities between organisms shown in Figure 12 (with the exception of *Thalassobacillus* sp.) supports the hypothesis that matrix effects control the biological influence on neodymium.

Because of the complex nature of the brine-microorganism suspensions, there is more than one possible explanation for our observations, and it is even more likely that a combination of these explanations provides the answer. Brine ionic strength, composition, and pH can affect Nd solubility and speciation but can also affect cell surface structures and integrity. These factors will be discussed below.

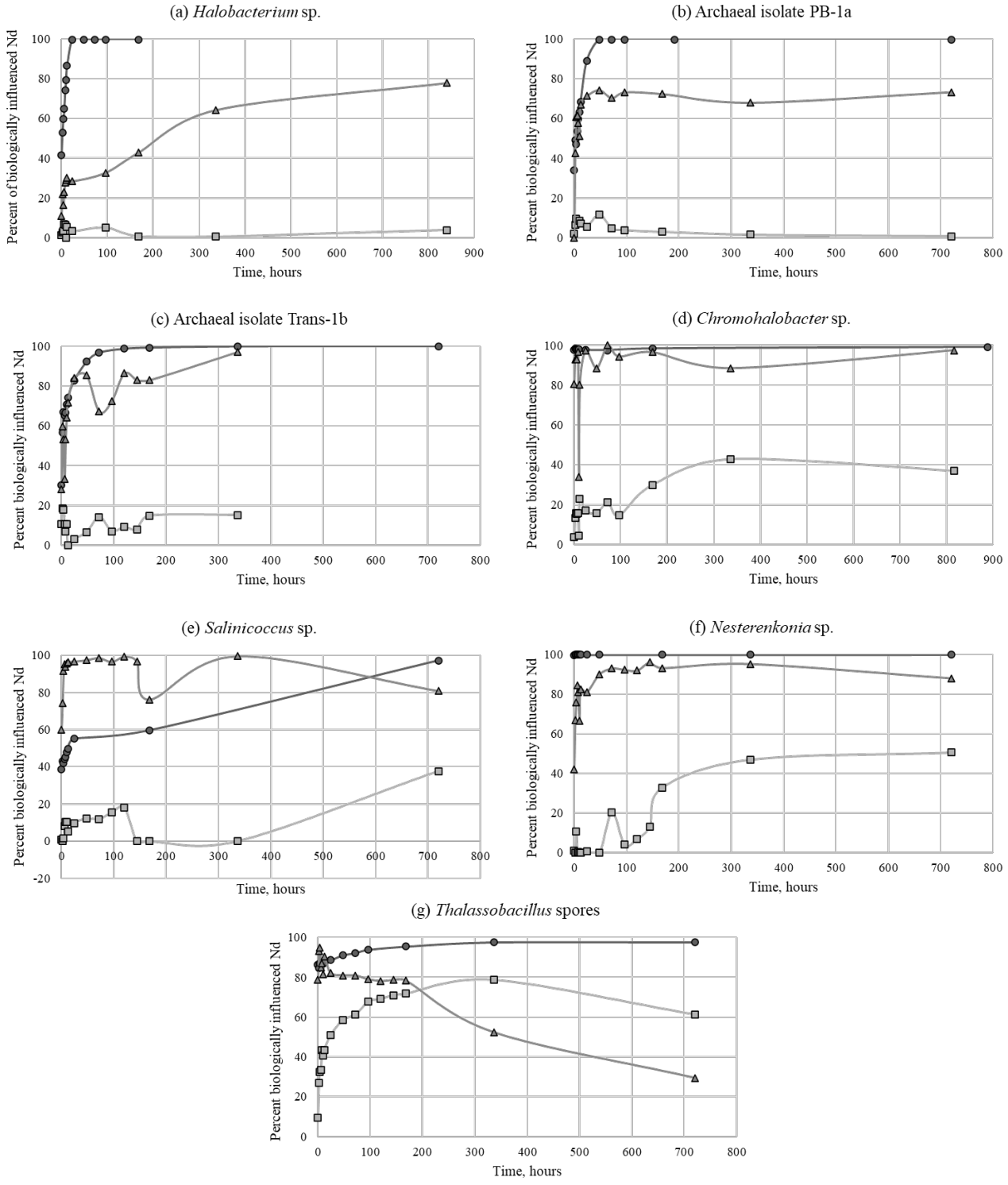


Figure 12. All tested organisms in the three tested matrices: circles = NaCl, squares = GWB, triangles = ERDA

4.4.1 Experimental starting neodymium concentrations exceeded solubility

The test concentrations of Nd in WIPP brines (0.2 μM) were chosen based on the solubility of Nd in each brine at its appropriate pC_{H+} value and on the detection limit for ICP-MS after the necessary sample dilution. Neodymium solubility is slightly lower in ERDA than in GWB (1.7×10^{-7} M versus 3×10^{-7}; Borkowski, 2009), and is also slightly less than the added target. This might explain the continued loss of Nd in abiotic solutions, with the exception of the *Halobacterium* sp. experiments. Nevertheless, this loss does not affect bioassociation calculations, except at final timepoints where the abiotic values were also zero.

4.4.2 Neodymium speciation

Experiments in simple NaCl solutions were conducted at pH ~ 6 to minimize the effects of hydrolysis and carbonate. In those experiments, the majority of Nd was present as Nd^{3+} and $NdCl^{2+}$ (M. Richmann, EQ3/6 calculations in Swanson et al., 2021; Migdisov and Williams-Jones, 2002). Both charged species could be involved in surface complexation. In experiments in WIPP brines, the pH was not adjusted. At brine pC_{H+} values and in the absence of carbonate, Nd is expected to exist as the hydrolysis species $Nd(OH)_2^+$, and to a lesser extent $Nd(OH)^{2+}$ and $Nd(OH)_3(aq)$ (Borkowski, 2009; Neck et al., 2009). These species are also charged, suggesting that surface complexation is possible. However, this does not explain the differences in apparent sorption between ERDA and GWB.

4.4.3 Loss of S-layers

The S-layer helps to maintain cell structure in haloarchaea. Magnesium stabilizes S-layers, and changes in Mg concentration can lead to S-layer loss and subsequent changes in cell morphology (Horikoshi et al., 1993). The requirement for Mg varies between organisms, but a *Haloarcula* species was found to change morphology below 41 mM. The test organisms for these experiments are usually grown in broth containing 98 mM of Mg. Ninety-percent ERDA contains 17 mM Mg, and 90% GWB contains 900 mM, suggesting that S-layer loss was possible in ERDA but unlikely in GWB.

EDTA concentrations as low as 10 mM can also lead to S-layer shedding due to chelation of S-layer Mg^{2+} ions (Cohen et al., 1991; Dyall-Smith, 2009). While WIPP inventory does not contain this much EDTA, it is unknown what effect a lower concentration could have.

In these experiments, loss of the S-layer should have led to a decrease in surface sites, and a change in cell morphology to small coccoid cells would have resulted in less surface area overall, ultimately in both cases resulting in less apparent sorption. Therefore, this is unlikely to be the sole reason for the observed findings for the haloarchaea.

4.4.4 Magnesium concentration

High concentrations of Mg will outcompete Nd for the limited number of available cell surface sites. A targeted investigation was conducted into the effects of magnesium concentration on Nd association with *Halobacterium* sp. and found that as concentrations increased, biological

influence decreased, suggesting less Nd sorbed (Figure 13; Swanson et al., 2023). At 1 M Mg (GWB concentration), *Halobacterium* sp. had less than 20 % influence on the Nd in solution.

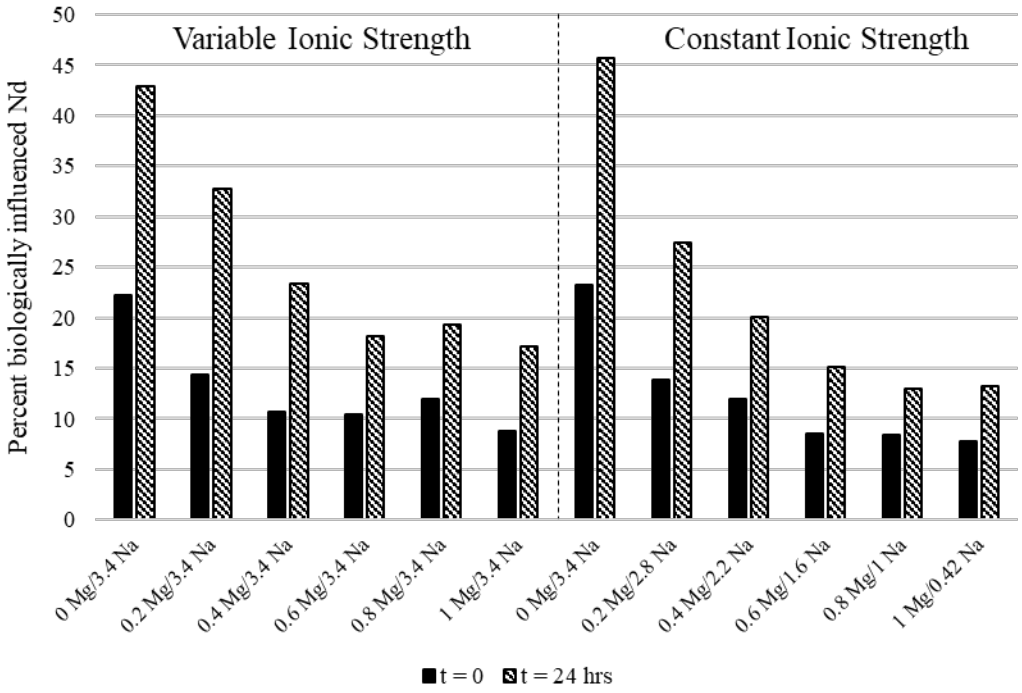


Figure 13. The effects of magnesium concentration on the percentage of biologically influenced Nd in solution at variable and constant ionic strengths, using *Halobacterium* sp. as the test organism (Swanson et al., 2023). Concentrations are given in M.

Lower association in GWB was observed with all tested organisms but was most noticeable in the extreme halophiles (Table 3). Regardless, the highest influence in GWB was with spores of *Thalassobacillus* sp. and still only reached 61% at the end of the experiment.

Table 3. Percent of biological influence on neodymium in GWB at the final time point for all tested organisms

Organism	Percent of Influence in GWB at Final Time Point
Halobacterium sp.	3.88
Isolate PB-1a	0.82
Isolate Trans-1b	15.2
Chromohalobacter sp.	37.0
Salinicoccus sp.	37.6
Nesterenkonia sp.	50.5
Thalassobacillus sp. spores	61.3

There is a relatively strong correlation between the above endpoints and the optimum concentration of NaCl for each organism. Since the less halophilic cells would experience osmotic shock more intensely than the extremely halophilic archaea, this could suggest some change in their cell surfaces or induced precipitation that would appear as association. For example, *Chromohalobacter* sp. previously shed its EPS layer, and Nd was found within its matrix (Swanson et al., 2023). However, this behavior was not observed in ERDA.

4.4.5 Biologically induced formation of Nd-containing precipitates

Biologically induced mineral precipitation is a well-known phenomenon, and many reviews on the mechanisms of this process exist in the literature (e.g., Frankel and Bazylinski, 2003; Hoffmann et al., 2021). Microbial cell surfaces offer numerous potential nucleation sites, while microbial activity (even if minimal) can often alter solution characteristics, such as pH, localized ion concentrations, and organic releases that can promote mineralization. In some cases, cell surface characteristics (e.g., EPS) enhance these conditions. Furthermore, in high ionic strength matrices, it can be easier to reach the saturation index for some ions. Biologically induced precipitation can result in loss of Nd from solution but would appear as apparent sorption in these experiments.

Some haloarchaea have been found to have a high density of carboxyl sites at their surface that catalyze dolomite precipitation under ideal Mg:Ca ratios, even under non-growth conditions (Kenward et al., 2013). Members of the *Chromohalobacter* and *Nesterenkonia* genera can precipitate different carbonate species, depending upon growth conditions (Rivadeneyra et al., 2000, 2006), and some can incorporate strontium into the mineral phase (Sánchez-Román et al., 2011), providing a possible radionuclide sink. Bader et al. (2019) found that resting cells of *Halobacterium noricense* DSM 15987 sequestered europium and curium through the release of phosphate species, one of which was a solid phase precipitate.

For some organisms, the S-layer also serves to protect cells from environmental toxicants, such as heavy metals (Chandramohan et al., 2019). If mineralization occurs on an S-layer, it can be shed and then precipitates (Schultze-Lam et al., 1996).

4.5 Basis for colloid enhancement parameter recommendations for CRA

The previous version of this report suggested that it might be easier to account for and predict long-term biological influence based on test matrix rather than on organism, given the differences in behavior in GWB. This is quite beneficial to PA, because it suggests that chemistry, more than biology, is the controlling factor for biocolloid measurements in the long-term. The new data support this hypothesis in most cases.

The results of these studies directly feed into calculations for the biocolloid parameters PROPMIC and CAPMIC. PROPMIC is the proportionality constant for the concentration of actinide associated with mobile microbes (defined as the moles of microbe-bound actinide divided by the moles of dissolved actinide). CAPMIC is the maximum concentration of actinide associated with mobile microbes (defined as the moles of microbe-bound actinide per liter).

Values for these parameters are significantly higher in all experiments conducted in simple NaCl than those in WIPP brines (Table 4; Figures 14 and 15). One reason for this difference could be the pH and composition of each matrix, which could affect Nd solubility. However, previous work in pC_{H+} 9.5-specific brine showed the same results as GWB, suggesting that pH is not the reason (Swanson, 2019). Another reason could be brine complexity and the potential for other interactions with Nd besides cell surfaces. Since both GWB and pC_{H+} 9.5-specific brines are high in Mg, this again suggests cation competition for cell surface sites. Since ERDA is mostly NaCl, it makes sense that bioassociation behavior should be more similar in ERDA and simple NaCl solutions. Taken together, these data point toward the background matrix as the dominant cause of the observed trends in these experiments.

Table 4 provides the median values for each data set (PROPMIC and CAPMIC), as well as the value marking the upper quartile (i.e., 75% of data fall below this value). These are calculated at three time points in three different matrices. Figures 14 and 15 show the spread in PROPMIC and CAPMIC calculations, which are best visualized in box and whisker plots.

Table 4. Comparison between the medians and upper quartiles of the data sets for PROPMIC and CAPMIC in each matrix. Calculations were made across 3 timepoints.

Time	Function	PROPMIC			CAPMIC		
		GWB	ERDA	NaCl	GWB	ERDA	NaCl
24-hour	median	0.07	4.22	7.74	5.39E-09	6.06E-08	3.81E-06
	upper quartile	0.19	5.25	35.9	6.23E-08	7.13E-07	6.92E-06
1 week	median	0.13	3.42	16.9	8.27E-09	7.75E-08	3.08E-06
	upper quartile	0.35	7.10	81.8	2.88E-08	2.34E-06	1.88E-05
final	median	0.26	3.52	41.2	1.04E-08	5.43E-08	2.39E-06
	upper quartile	0.60	5.81	83.6	3.45E-06	2.50E-06	6.29E-06

These findings support DOE's argument that the data feeding these parameters be taken from experiments conducted under more WIPP-relevant conditions, i.e., in WIPP brines. As is clear in Figures 14 and 15, using NaCl only as the background matrix skews these values upward, thus it is not practical to use values obtained from the literature.

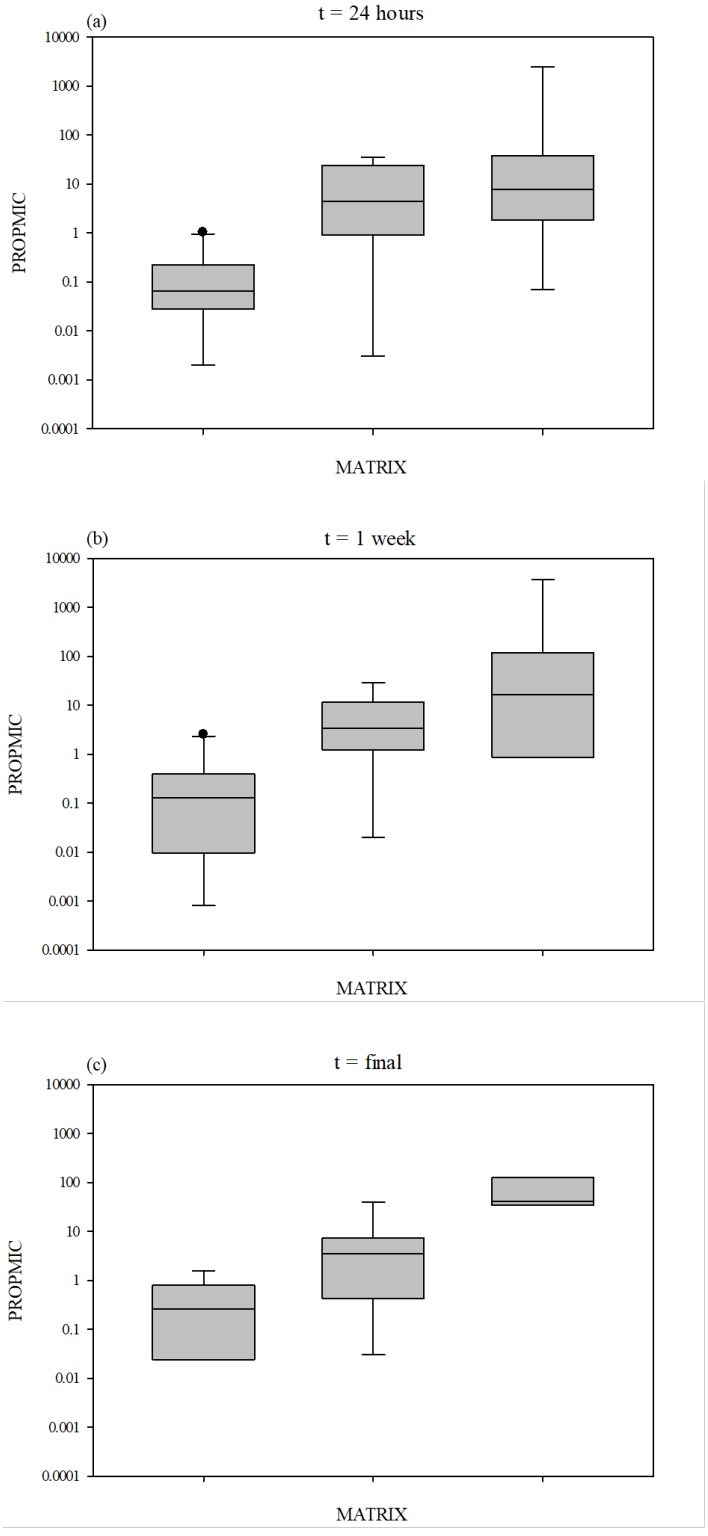


Figure 14. Box and whisker plots summarizing PROPMIC data sets and showing outlying data for values calculated at (a) 24 hours, (b) 1 week, and (c) final timepoints. GWB (left box), ERDA (center box) and NaCl (right box).

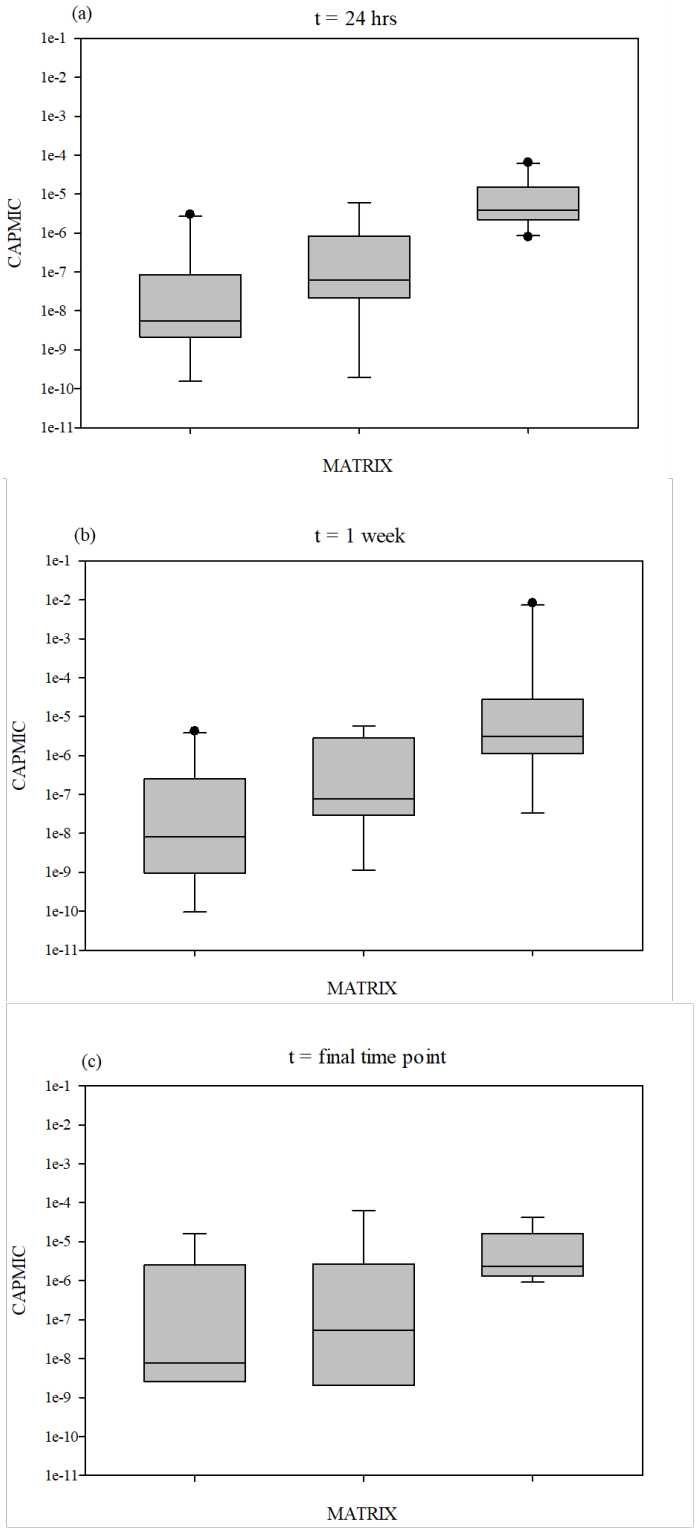


Figure 15. Box and whisker plots summarizing CAPMIC data sets and showing outlying data for values calculated at (a) 24 hours, (b) 1 week, and (c) final timepoints. GWB (left box), ERDA (center box) and NaCl (right box).

Future work will continue to focus on determining the mechanisms of biological influence (e.g., biomineralization, EPS sorption, etc.) as well as new tests with a different oxidation state (e.g., An(V)). However, a full mechanistic understanding for An(III) is not necessary for generating the colloid parameters, and the data presented in this report can be used to update PROPMIC and CAPMIC values for the upcoming CRA.

5.0 QUALITY ASSURANCE, DATA TRACEABILITY AND DOCUMENTATION

The data presented in this report were generated as Quality Level 1 data, in accordance with the CBFO Quality Assurance Program Document. Experiments were performed under the test plan, "Microbial Interactions in the Waste Isolation Pilot Plant" (LCO-ACP-12). Descriptions of the experiments can be found in the scientific notebooks, "Sorption of An(III) and An(IV) Actinides or Analogues to Halophilic Microorganisms under WIPP-Relevant Conditions" (TIP-012-3/4, 3/5, and 3/6). Preliminary supporting information can be found in the developmental scientific notebooks "Microbiology for the WIPP" (BIO 1 and 2). The following data packages were generated to support this report. Packages submitted in support of previous reports are not included below, but their respective reports are referenced in this document.

- Neodymium-*Chromohalobacter* sp. Association Kinetics Experiment (ACP-2211-07-09-01)
- Neodymium-PB-1a sp. Association Kinetics Experiment (ACP-2209-02-09-02)
- Neodymium-*Salinicoccus* sp. Association Kinetics Experiment (ACP-2209-02-09-01)
- Neodymium-*Nesterenkonia* sp. Association Kinetics Experiment (ACP-2211-04-09-01)
- Neodymium-*Thalassobacillus* sp. Spores Association Kinetics Experiment (ACP-2211-07-09-10)
- Neodymium-*Chromohalobacter* sp. Association Kinetics Experiments in WIPP Brines (ACP-2211-07-09-07)
- Neodymium-PB-1a sp. Association Kinetics Experiments in WIPP Brines (ACP-2211-07-09-04)
- Neodymium-Trans-1b Association Kinetics Experiments in NaCl (ACP-2408-02-01-02)
- Neodymium-Trans-1b Association Kinetics Experiments in WIPP Brines (ACP-2408-03-01-02)

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7.0 APPENDIX

Neodymium measured in solution and the percent of biological influence for all tested organisms and their associated abiotic controls at 24 hours and final timepoints.

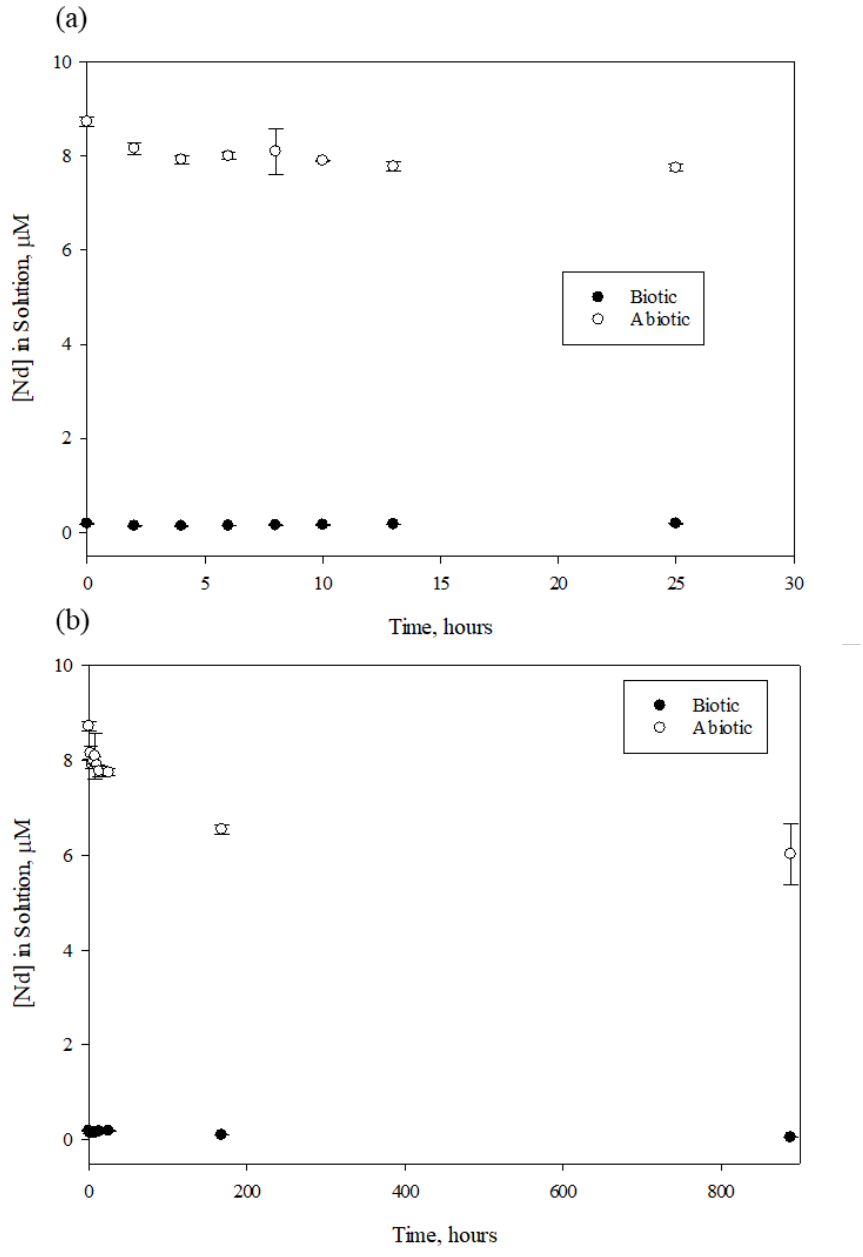


Figure A1. Concentrations of Nd in biotic and abiotic samples from *Chromohalobacter* sp. experiments in 2.6 M NaCl solution over a) 24 hours and b) ~ 5 weeks. Error bars represent the deviation from the mean of triplicate measurements.

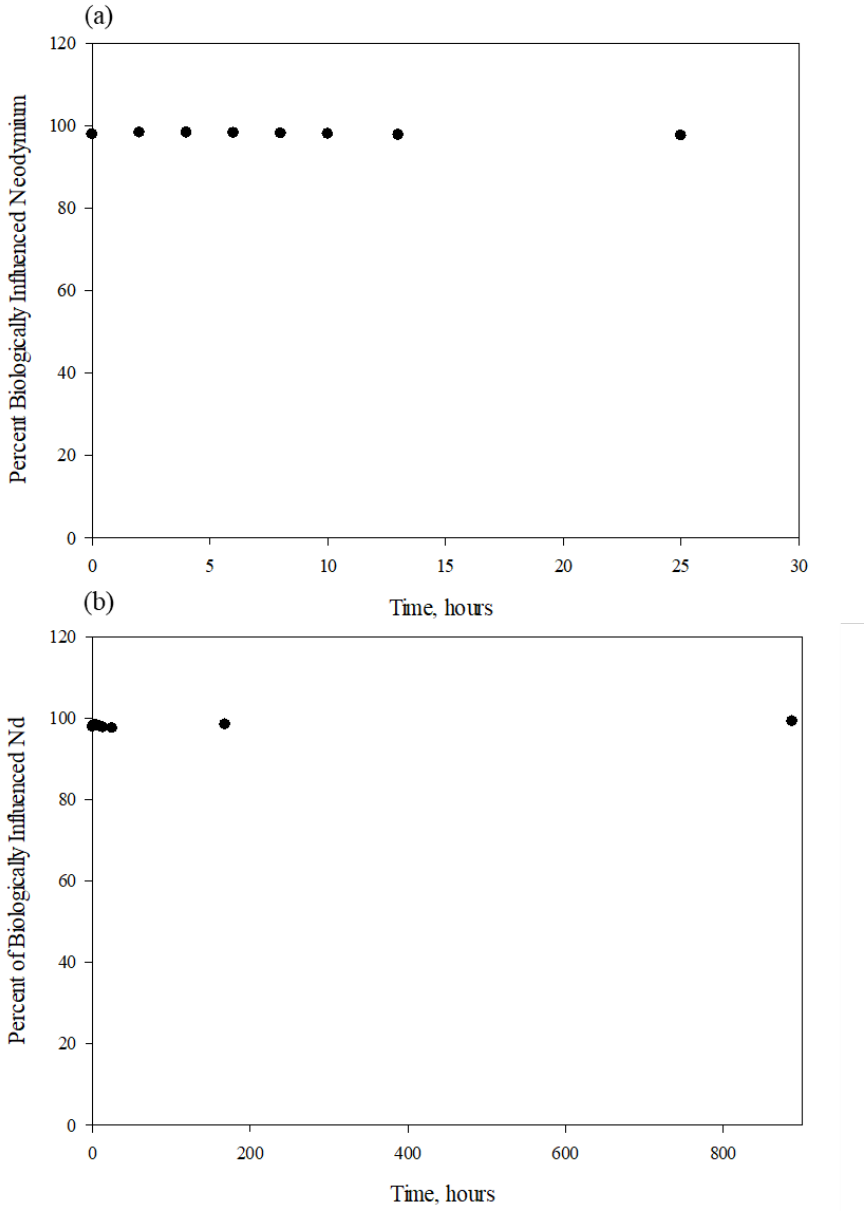


Figure A2. Percent of biologically influenced neodymium in 2.6 M NaCl of *Chromohalobacter* sp. experiments over a) 24 hours and b) ~ 5 weeks.

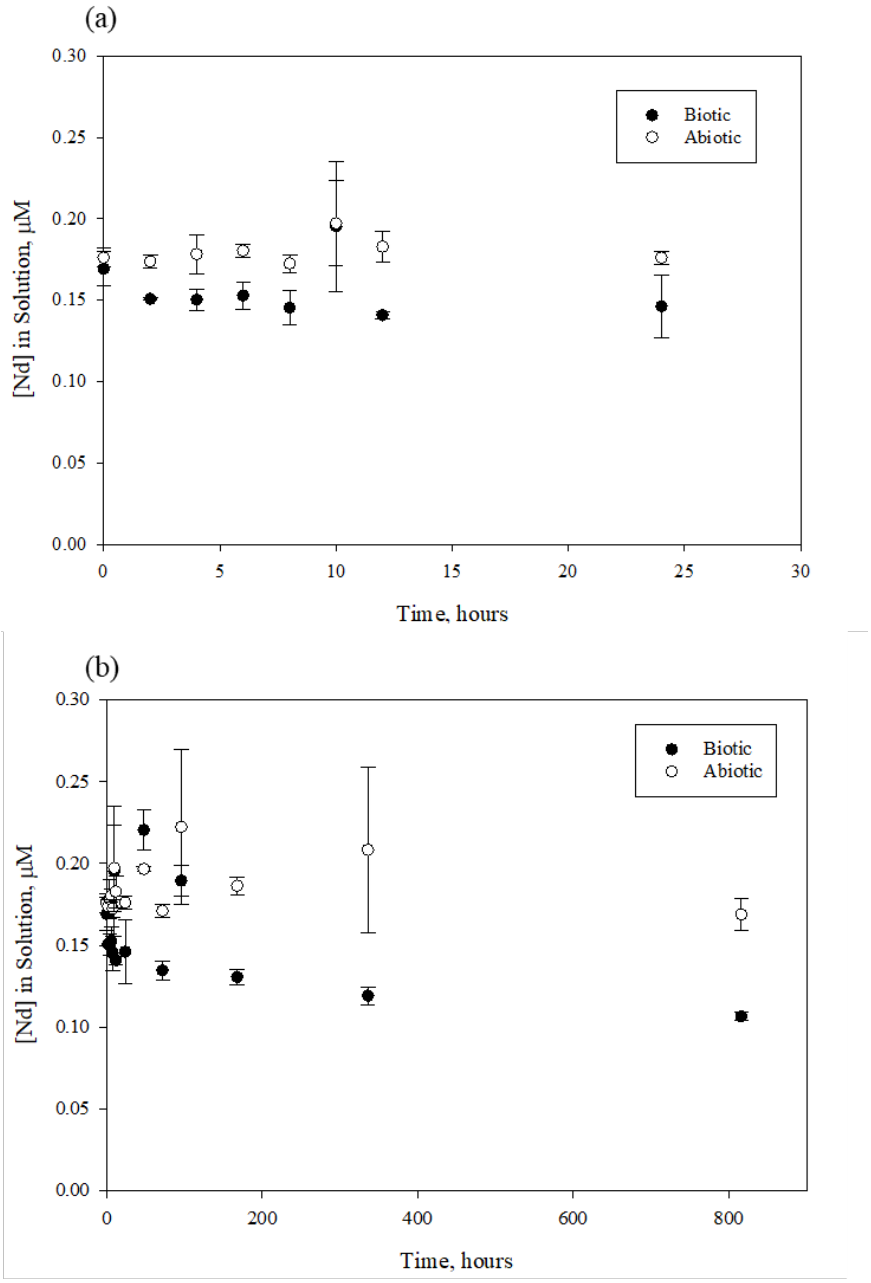


Figure A3. Concentrations of Nd in biotic and abiotic samples from *Chromohalobacter* sp. experiments in GWB over a) 24 hours and b) ~ 5 weeks. Error bars represent the deviation from the mean of triplicate measurements.

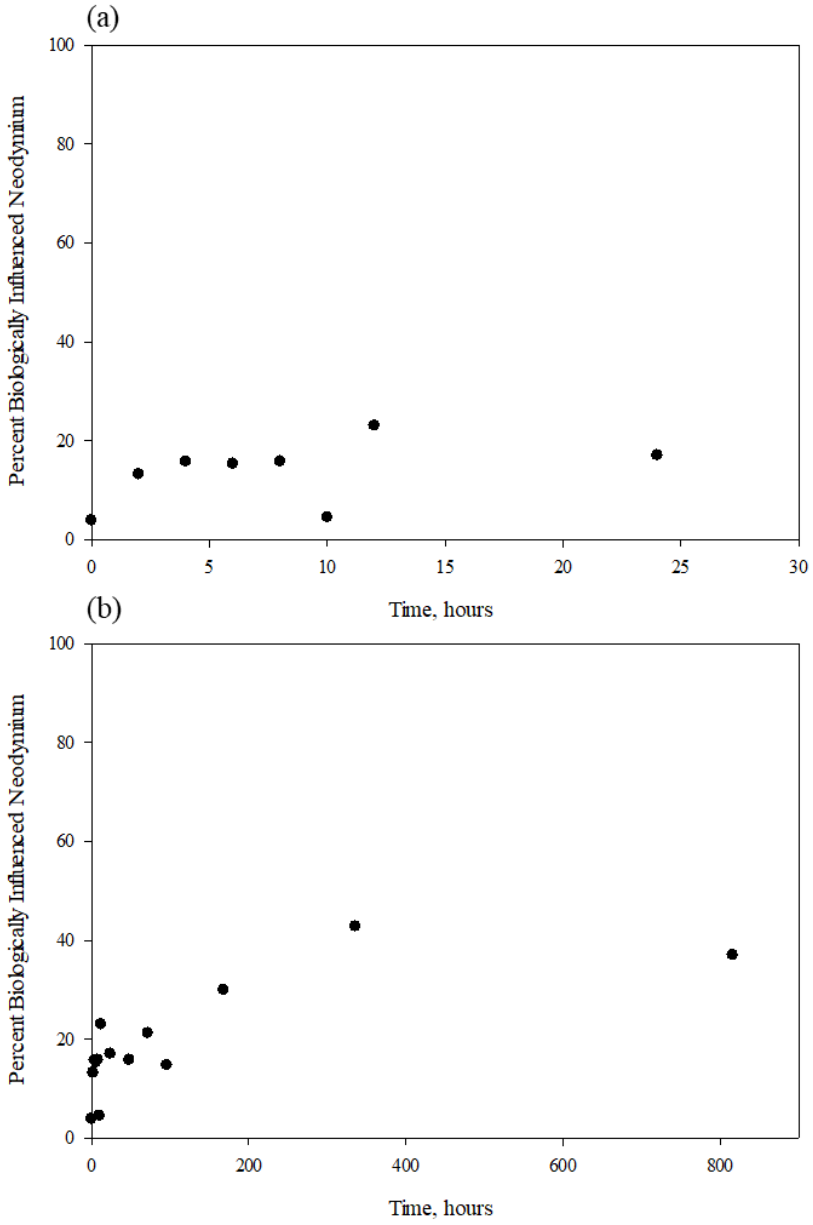


Figure A4. Percent of biologically influenced neodymium in GWB of *Chromohalobacter* sp. experiments over a) 24 hours and b) ~ 5 weeks.

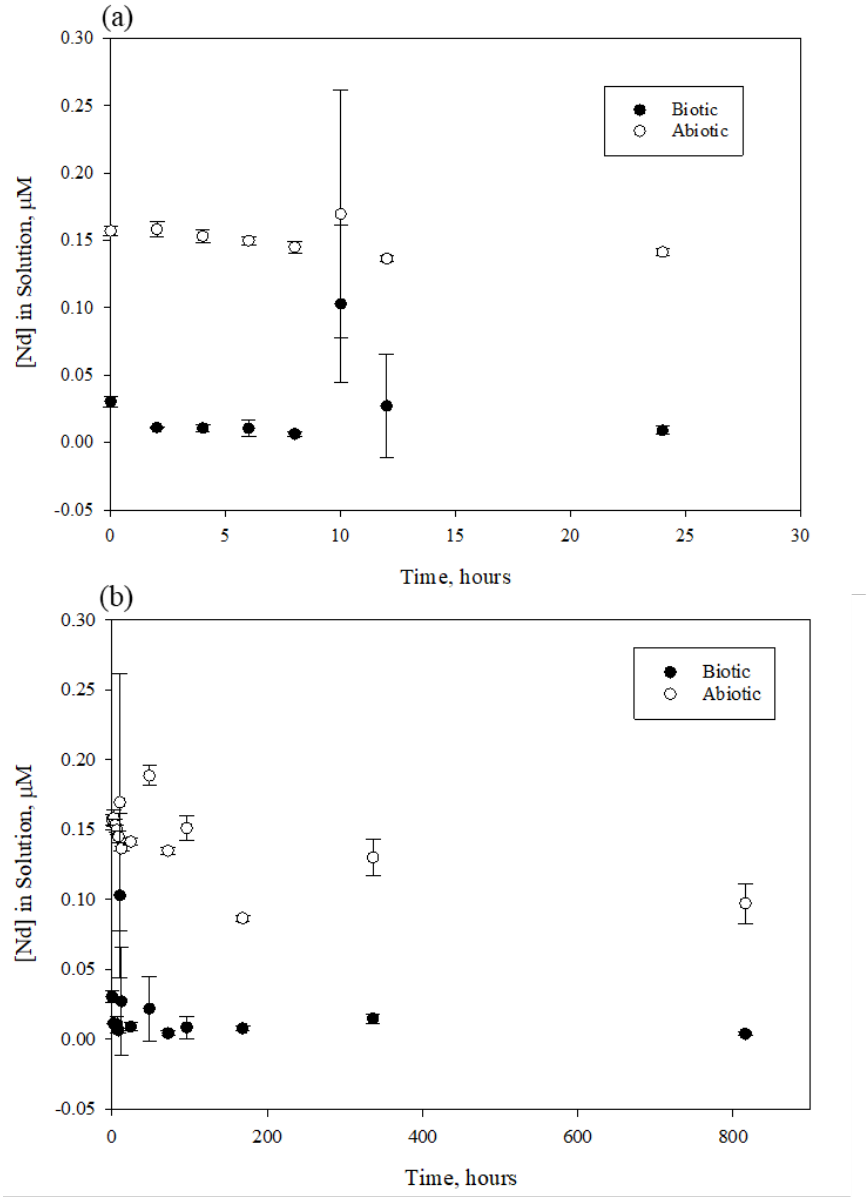


Figure A5. Concentrations of Nd in biotic and abiotic samples from *Chromohalobacter* sp. experiments in ERDA over a) 24 hours and b) ~ 5 weeks. Error bars represent the deviation from the mean of triplicate measurements.

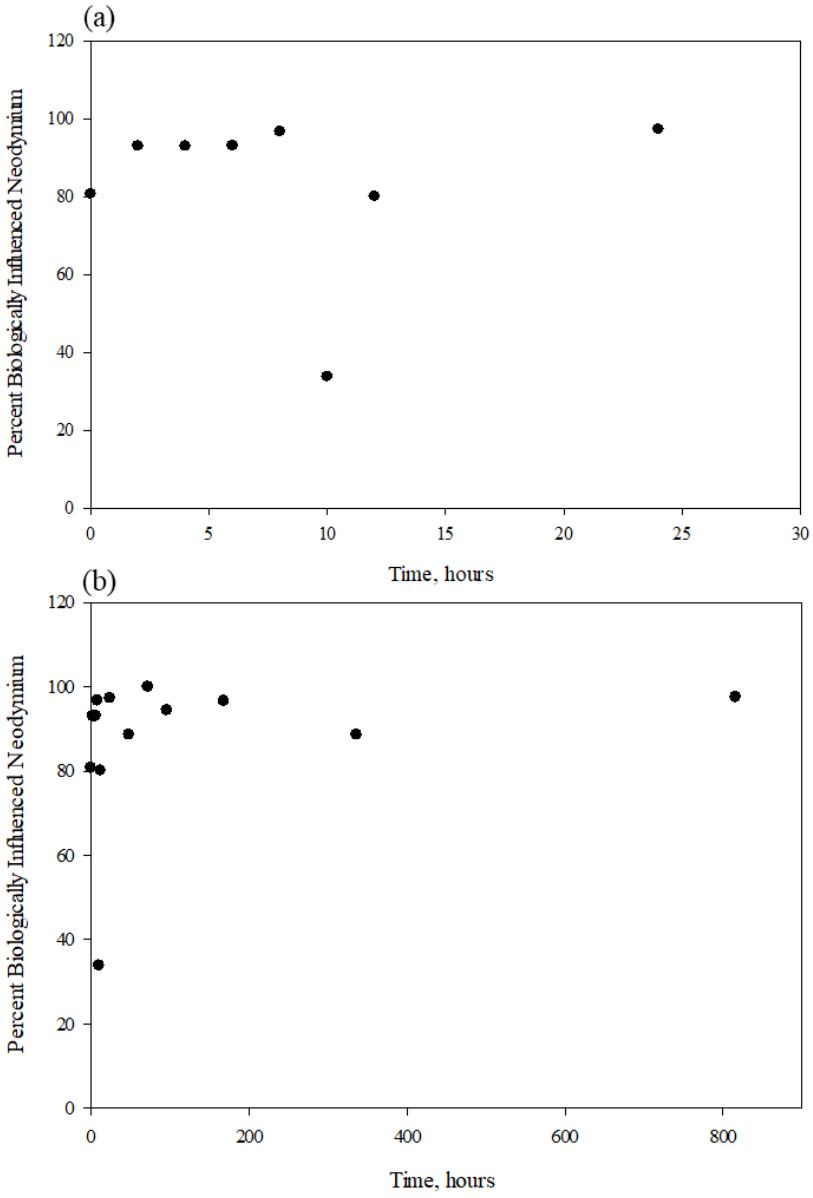


Figure A6. Percent of biologically influenced neodymium in ERDA of *Chromohalobacter* sp. experiments over a) 24 hours and b) ~ 5 weeks.

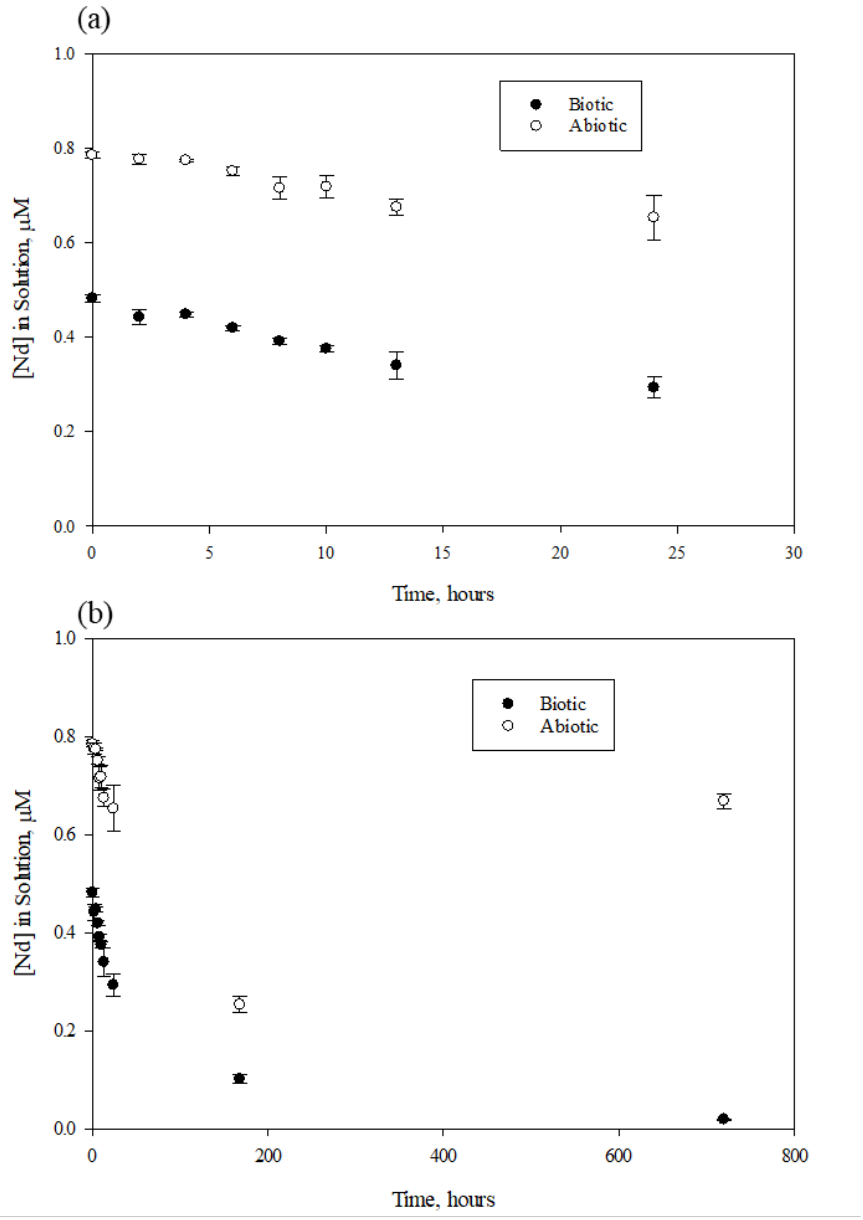


Figure A7. Neodymium concentration in biotic and abiotic 1.7 M NaCl solutions from *Salinicoccus* sp. experiments over a) the first 24 hours and b) one month. Error bars represent the deviation from the mean of triplicate samples.

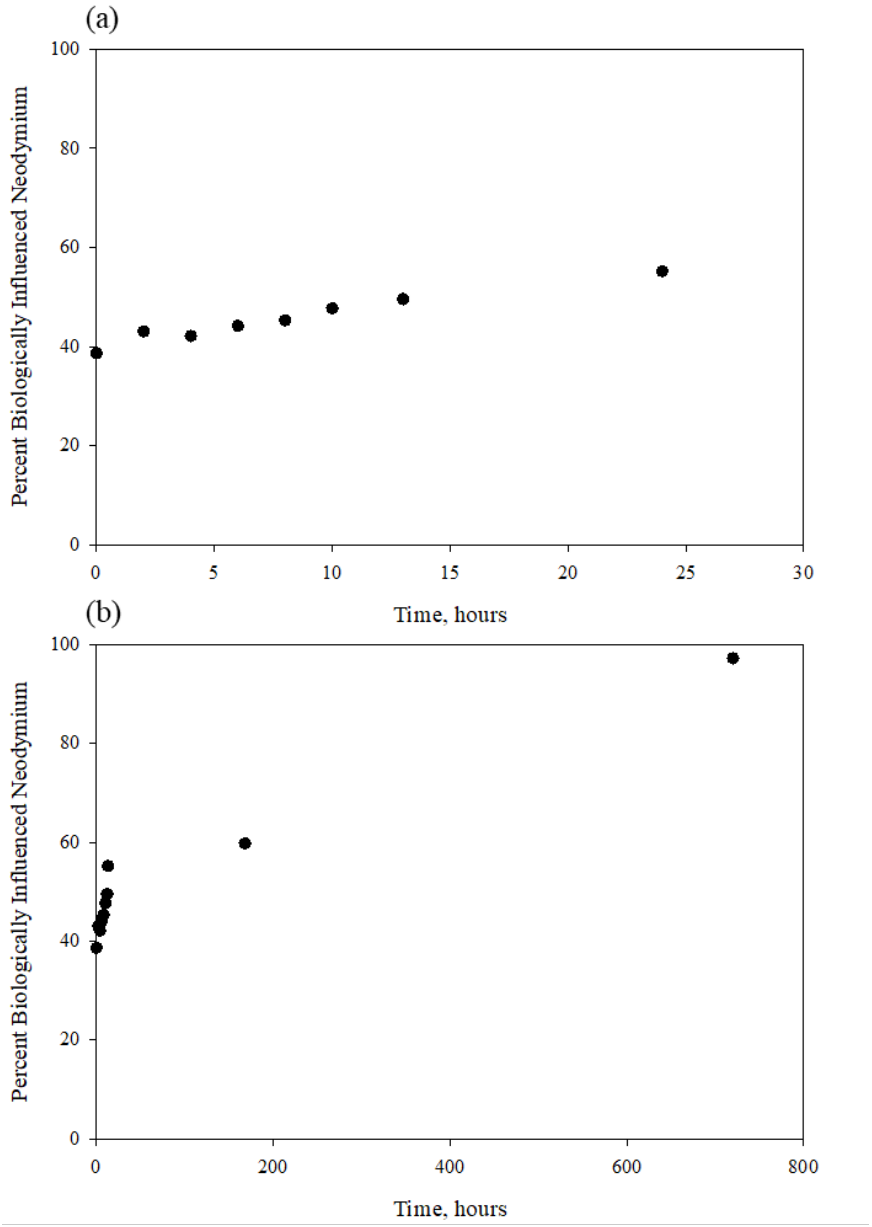


Figure A8. Percent of biologically influenced neodymium in 1.7 M NaCl of *Salinicoccus* sp. experiments over one month.

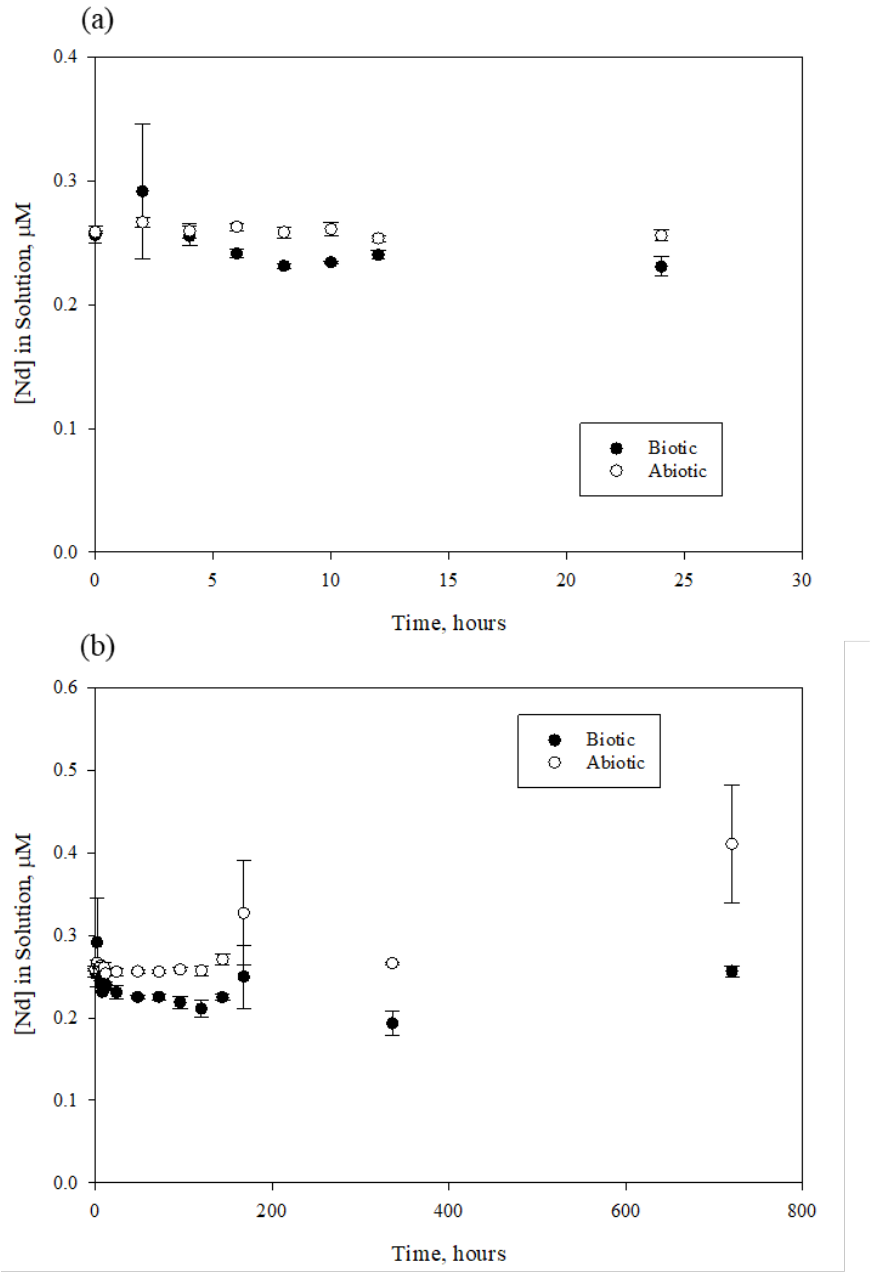


Figure A9. Concentrations of Nd in biotic and abiotic samples from *Salinicoccus* sp. experiments in GWB over a) 24 hours and b) one month. Error bars represent the standard deviation of triplicate measurements.

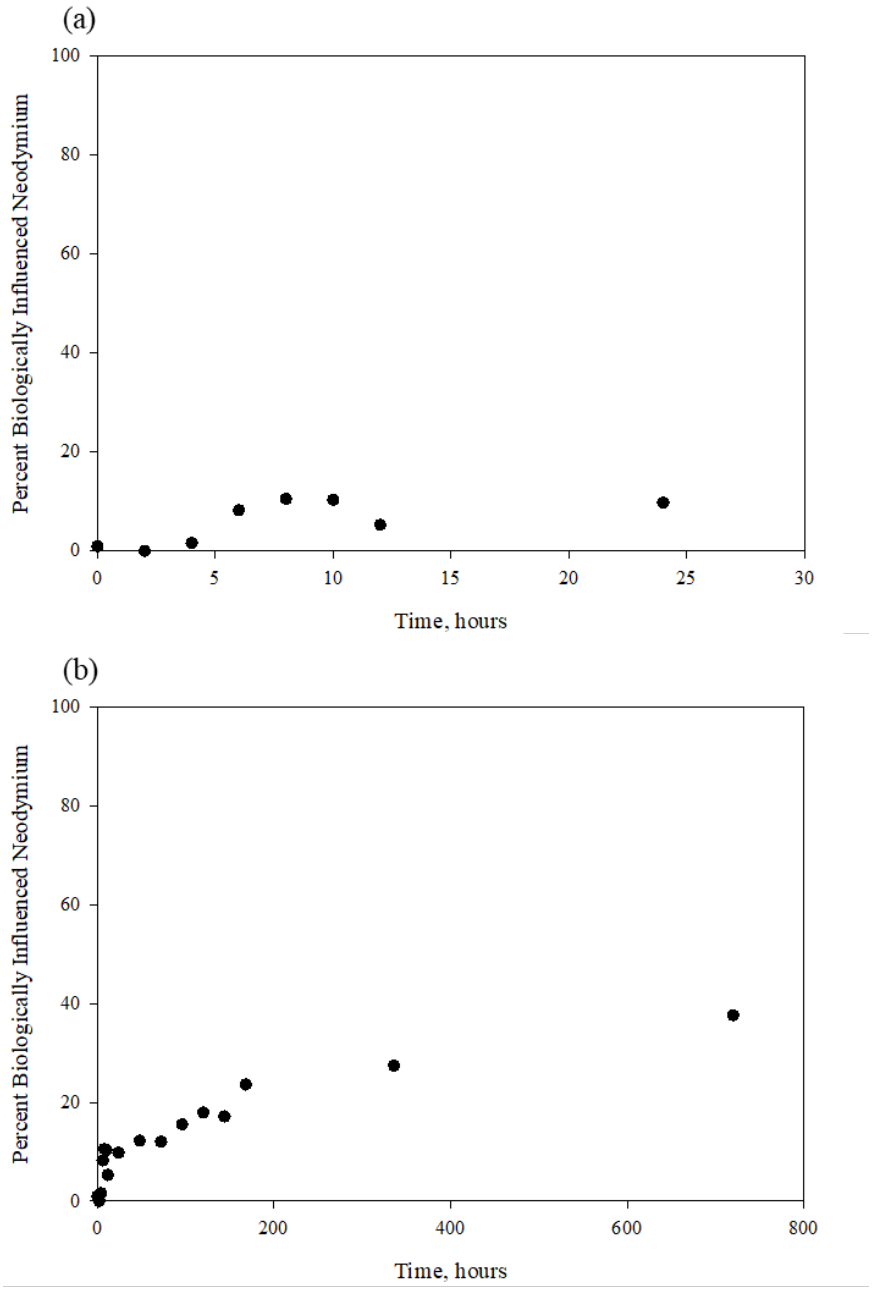


Figure A10. Percent of biologically influenced neodymium in GWB of *Salinicoccus* sp. experiments over a) 24 hours and b) one month.

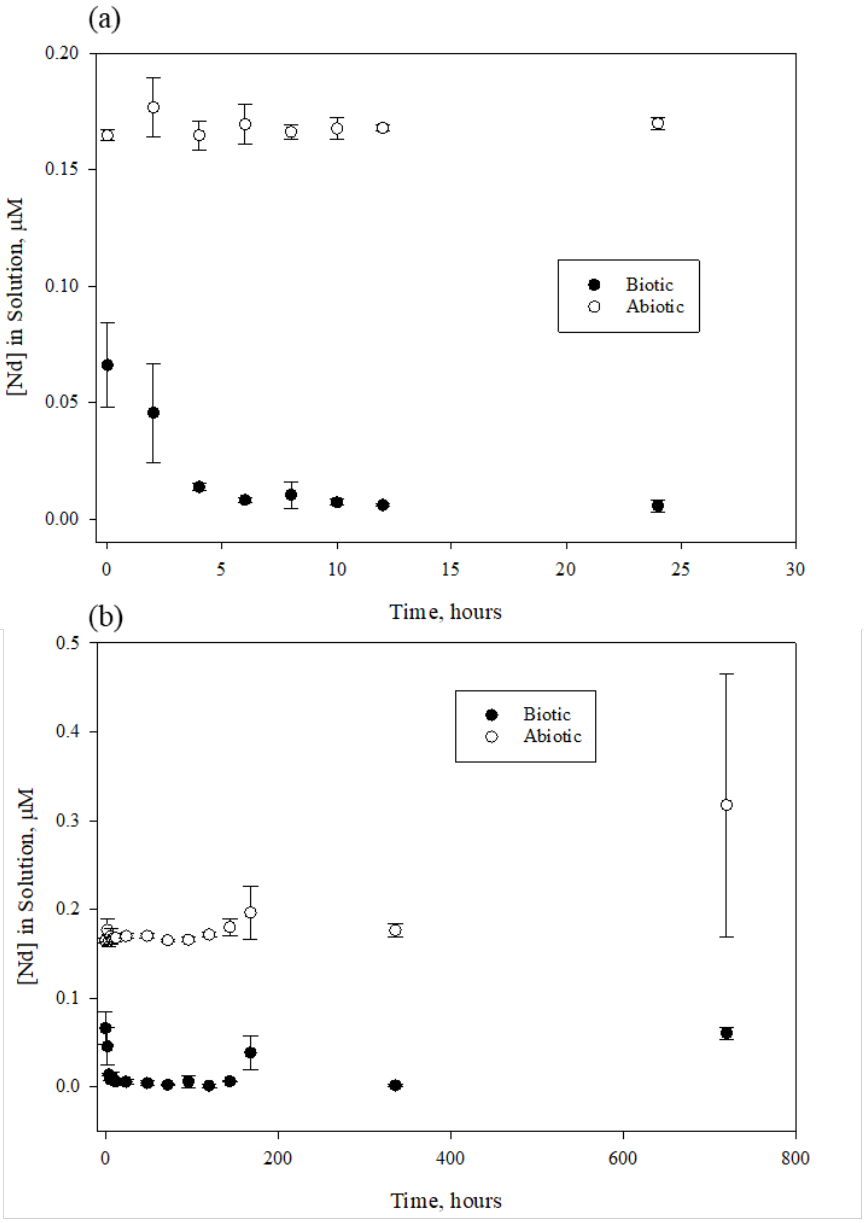


Figure A11. Concentrations of Nd in biotic and abiotic samples from *Salinicoccus* sp. experiments in ERDA over a) 24 hours and b) one month. Error bars represent the standard deviation of triplicate measurements.

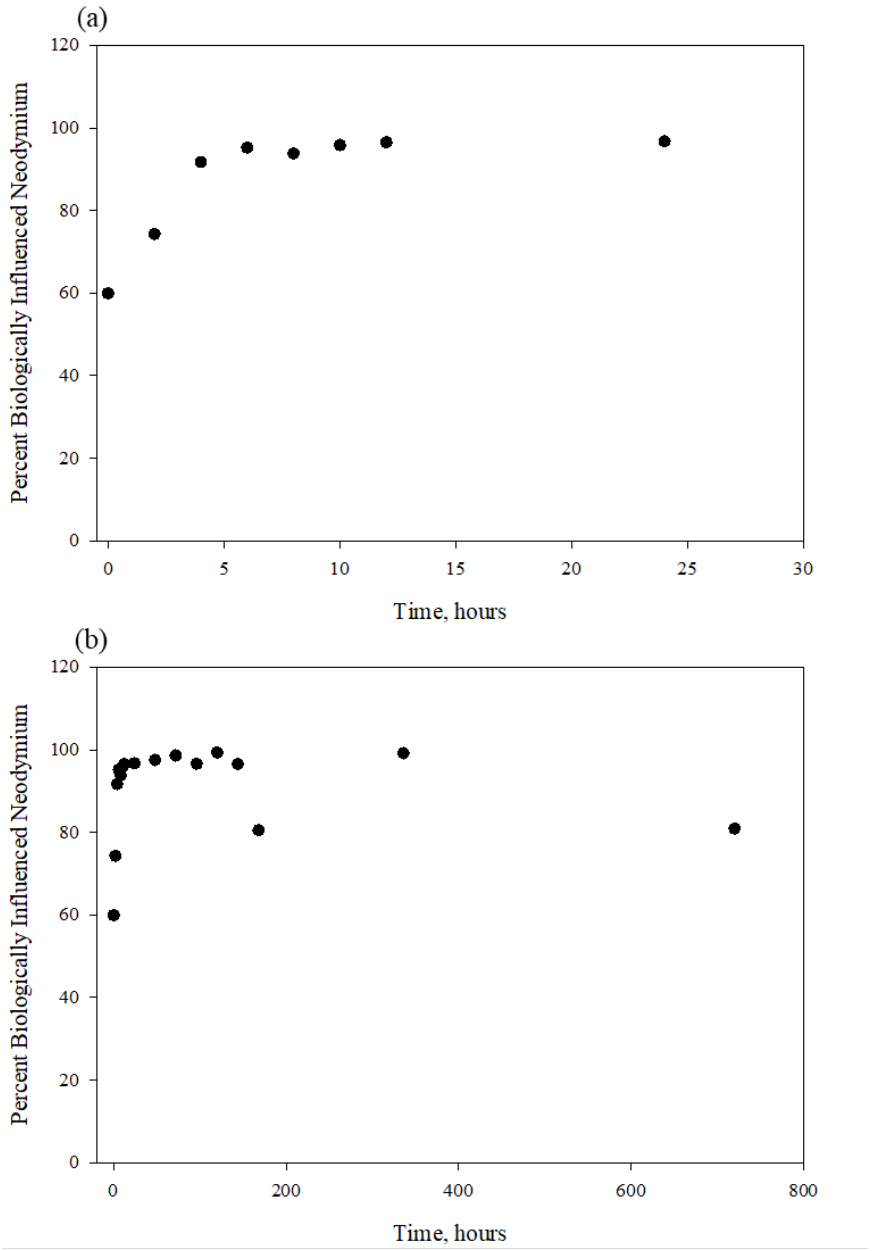


Figure A12. Percent of biologically influenced neodymium in ERDA of *Salinicoccus* sp. experiments over a) 24 hours and b) one month.

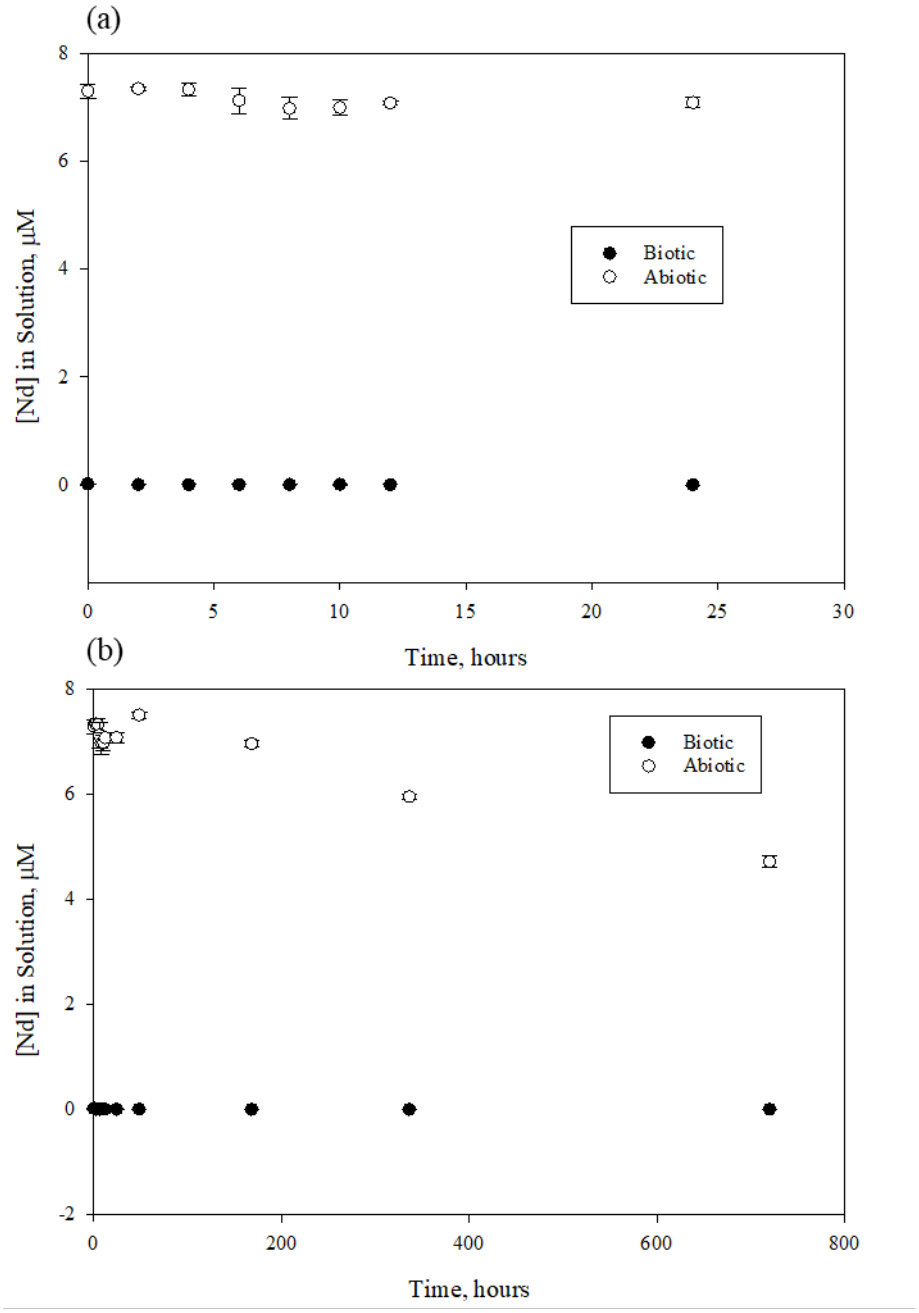


Figure A13. Concentrations of Nd in biotic and abiotic samples from *Nesterenkonia* sp. experiments in 0.4 M NaCl over a) 24 hours and b) one month. Error bars represent the deviation from the mean of triplicate measurements.

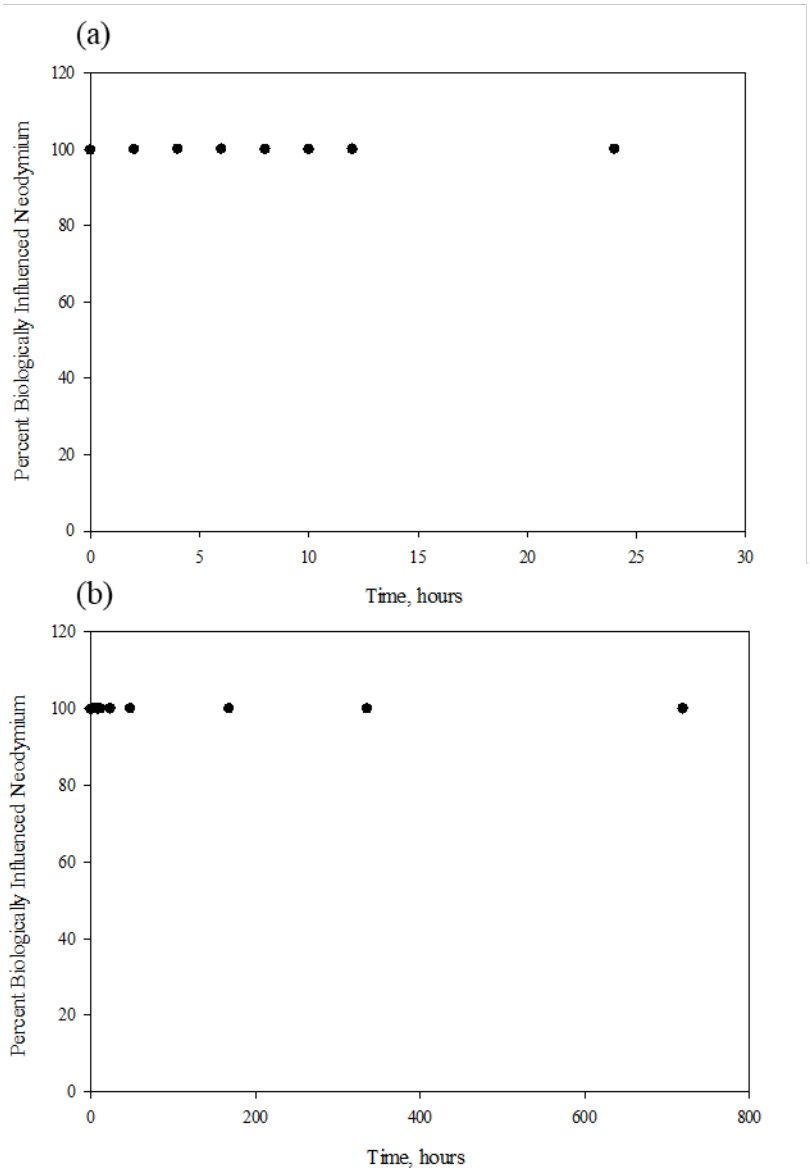


Figure A14. Percent of biologically influenced neodymium in 0.4 M NaCl of *Nesterenkonia* sp. experiments over a) 24 hours and b) one month.

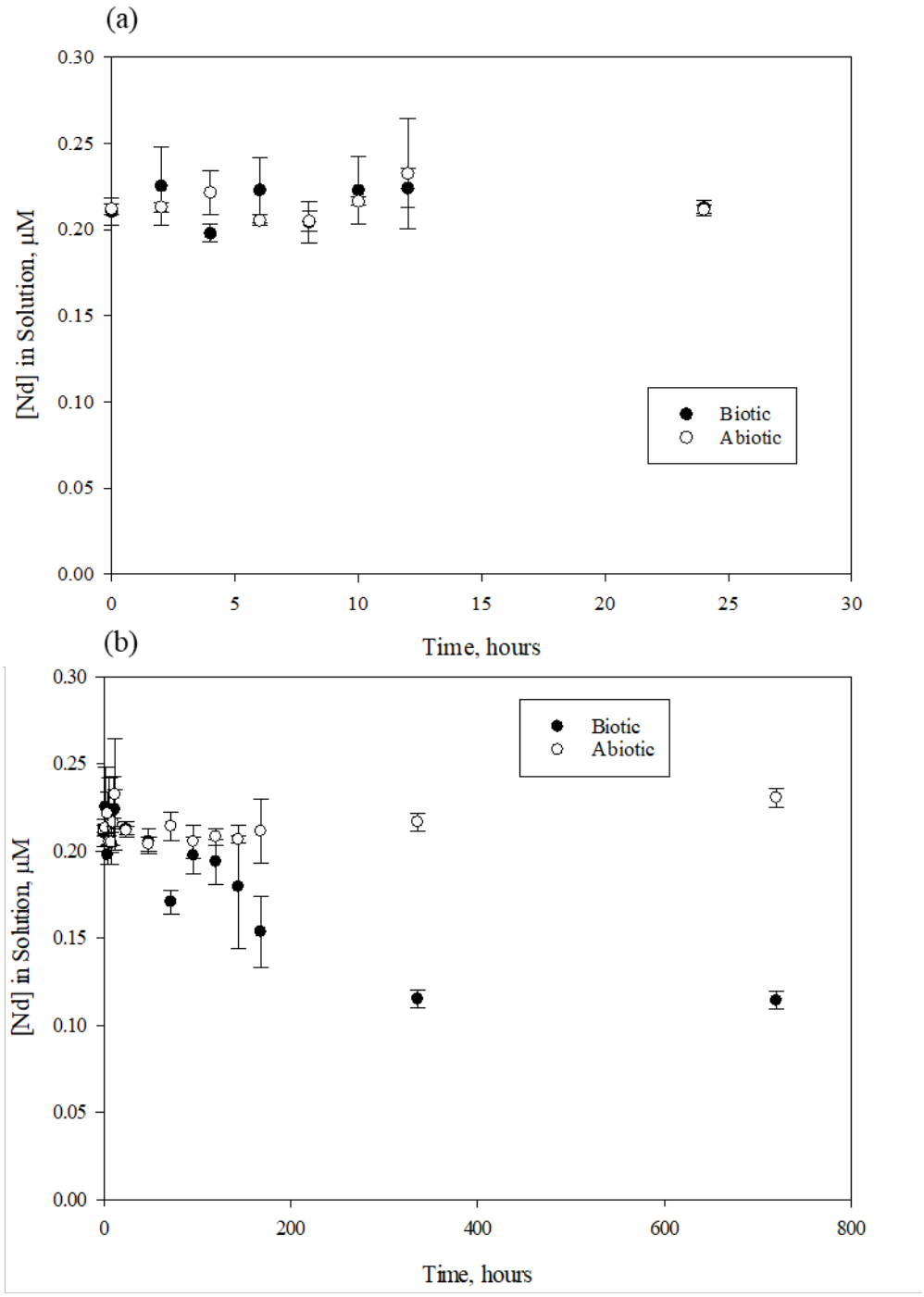


Figure A15. Concentrations of Nd in biotic and abiotic samples from *Nesterenkonia* sp. experiments in GWB over a) 24 hours and b) one month. Error bars represent the deviation from the mean of triplicate measurements.

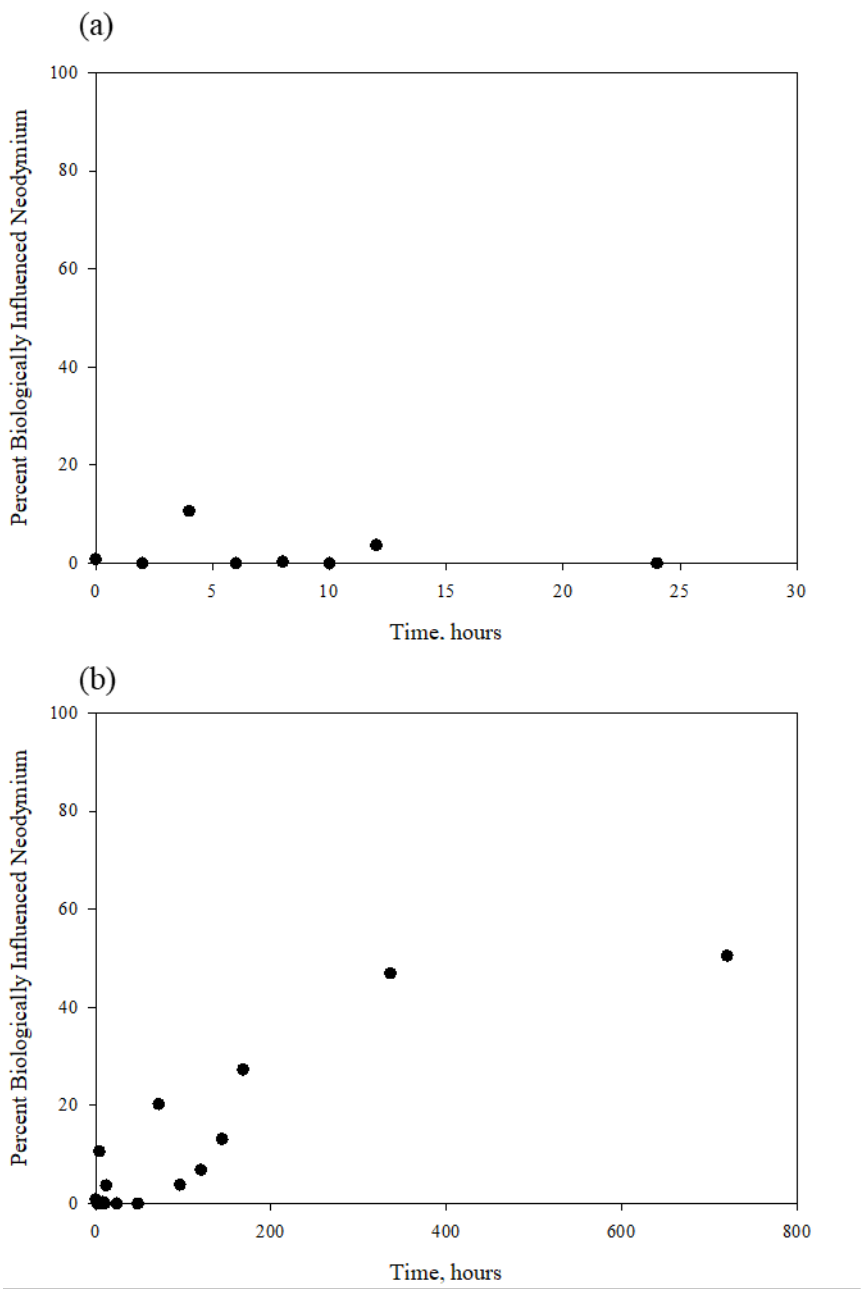


Figure A16. Percent of biologically influenced neodymium in GWB of *Nesterenkonia* sp. experiments over a) 24 hours and b) one month.

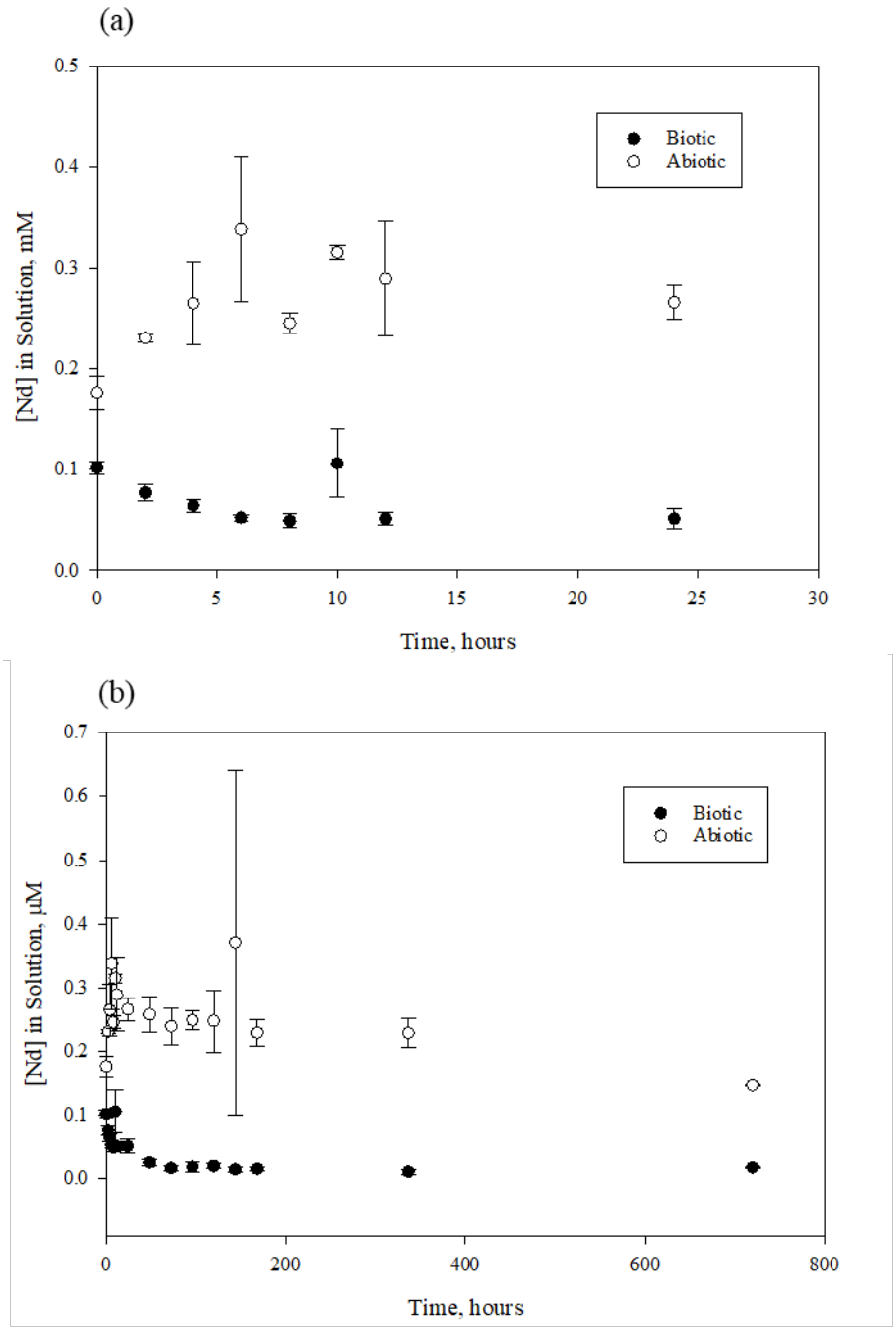


Figure A17. Concentrations of Nd in biotic and abiotic samples from *Nesterenkonia* sp. experiments in ERDA over a) 24 hours and b) one month. Error bars represent the deviation from the mean of triplicate measurements.

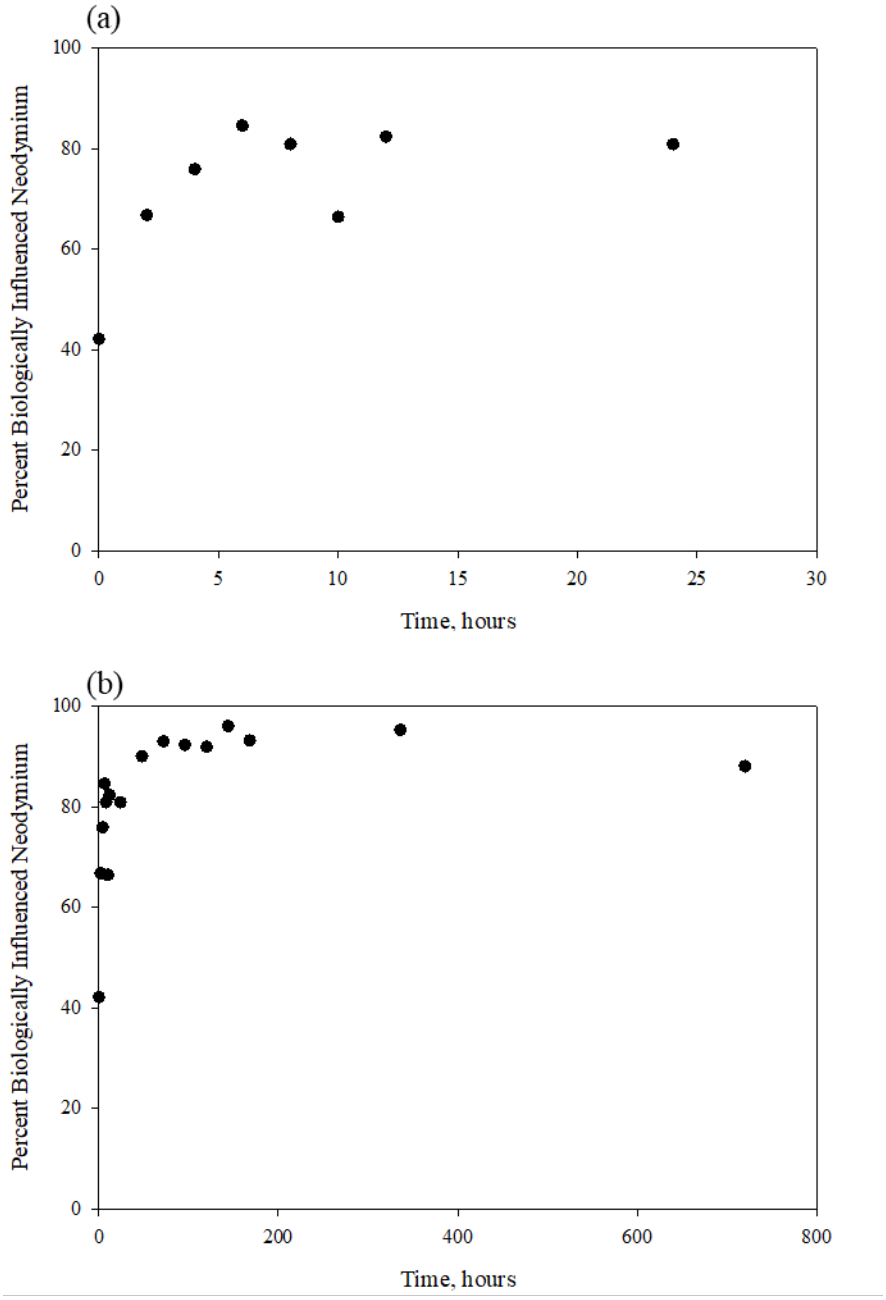


Figure A18. Percent of biologically influenced neodymium in ERDA of *Nesterenkonia* sp. experiments over a) 24 hours and b) one month.

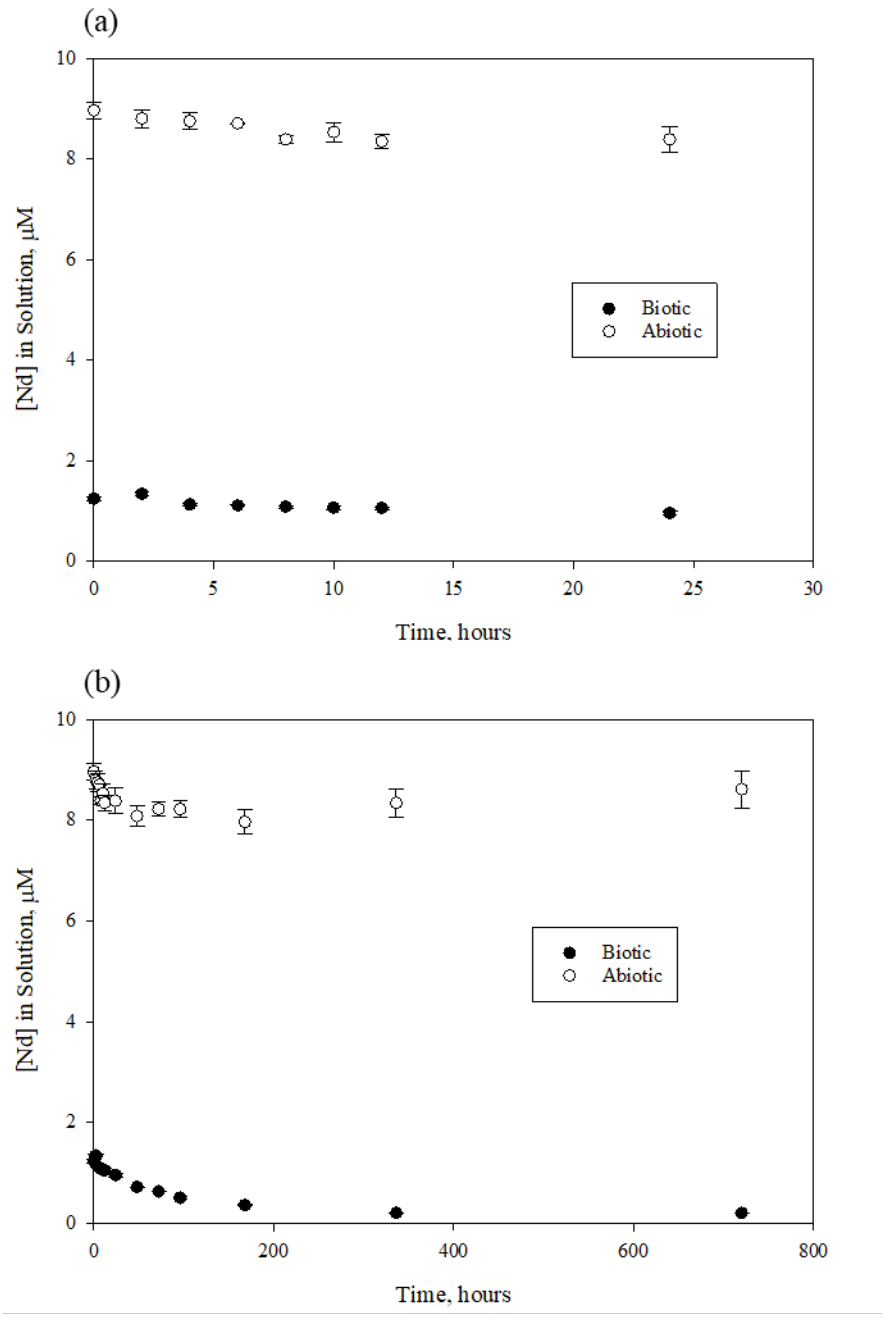


Figure A19. Concentrations of Nd in biotic and abiotic samples from *Thalassobacillus* sp. spore experiments in 0.76 M NaCl over a) 24 hours and b) one month. Error bars represent the deviation from the mean of triplicate measurements.

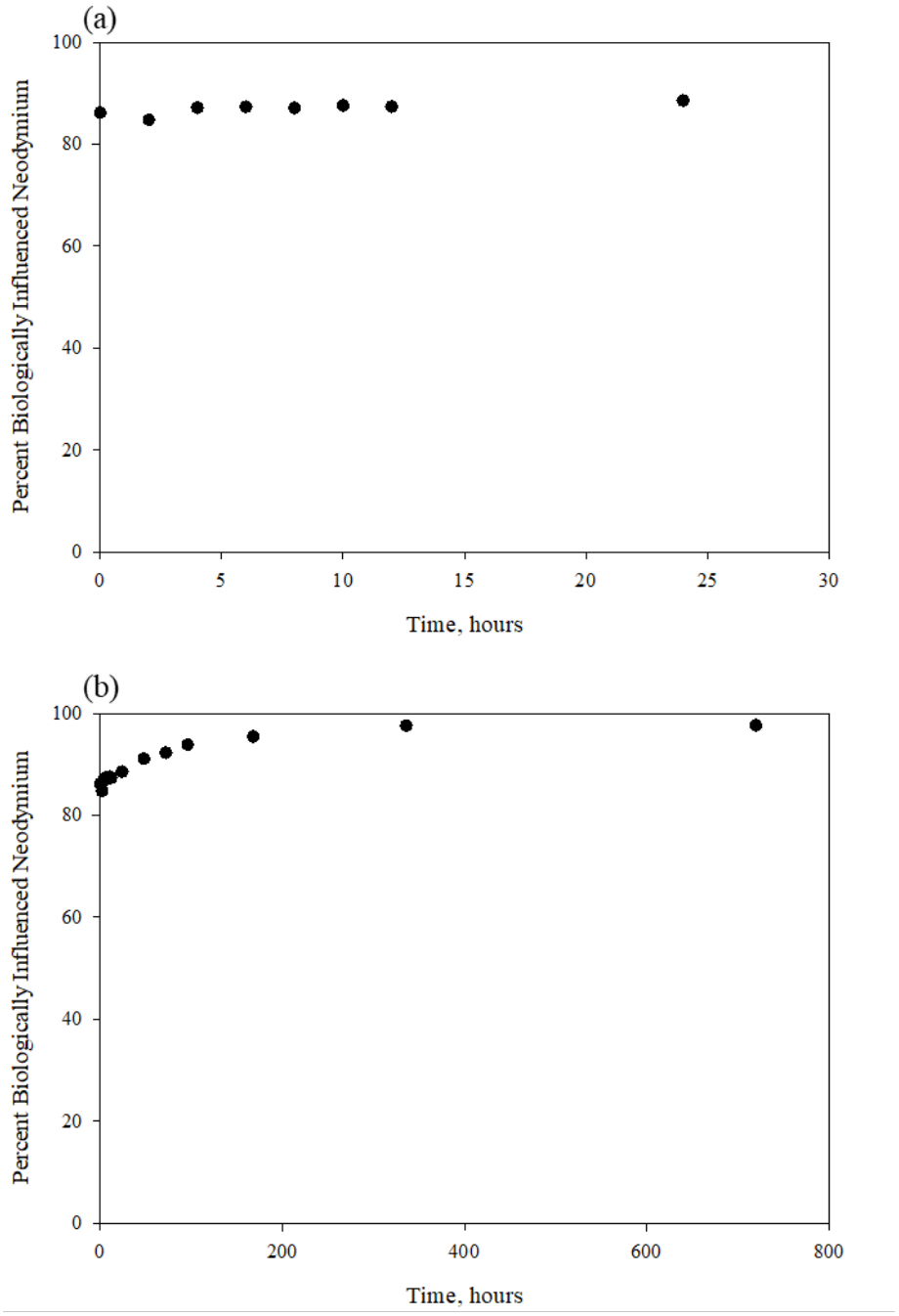


Figure A20. Percent of biologically influenced neodymium in 0.76 M NaCl of *Thalassobacillus* sp. spore experiments over a) 24 hours and b) one month.

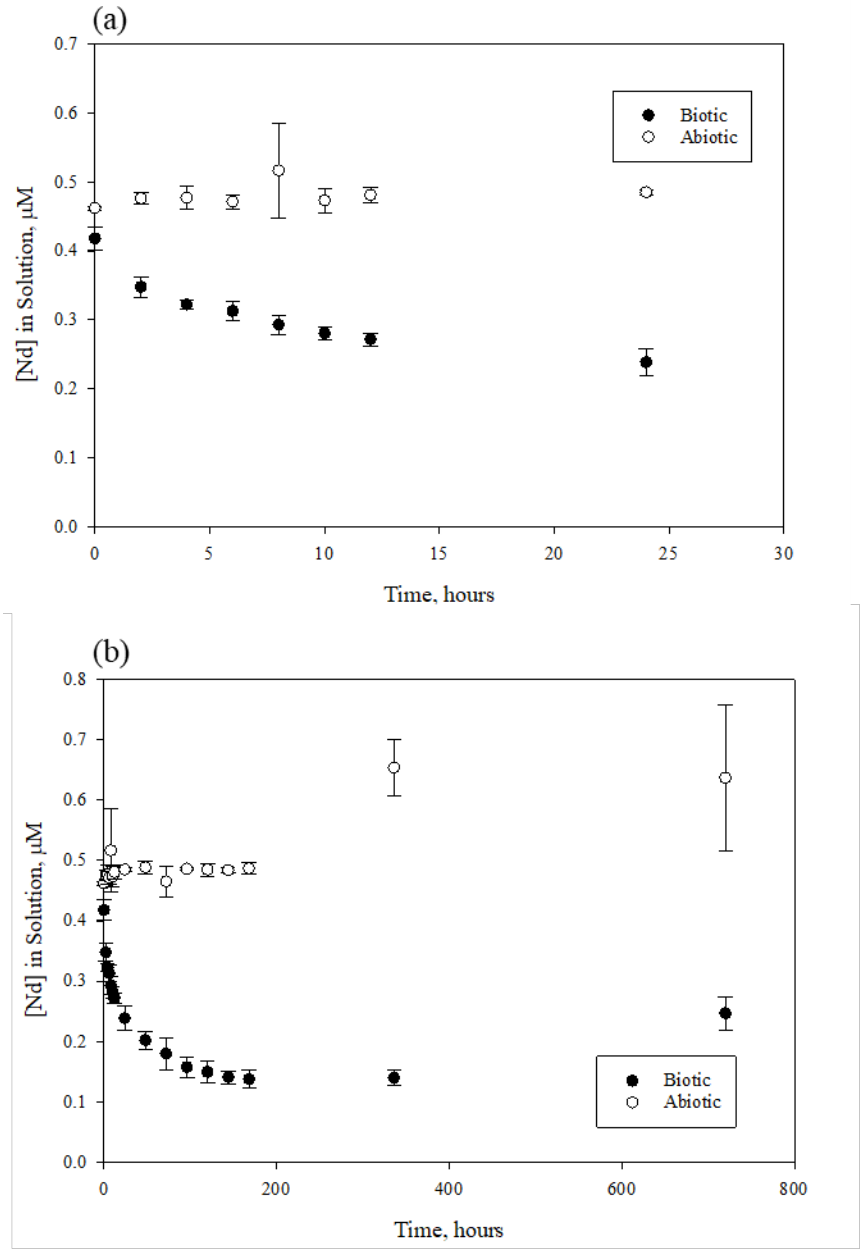


Figure A21. Concentrations of Nd in biotic and abiotic samples from *Thalassobacillus* sp. spore experiments in GWB over a) 24 hours and b) one month. Error bars represent the deviation from the mean of triplicate measurements.

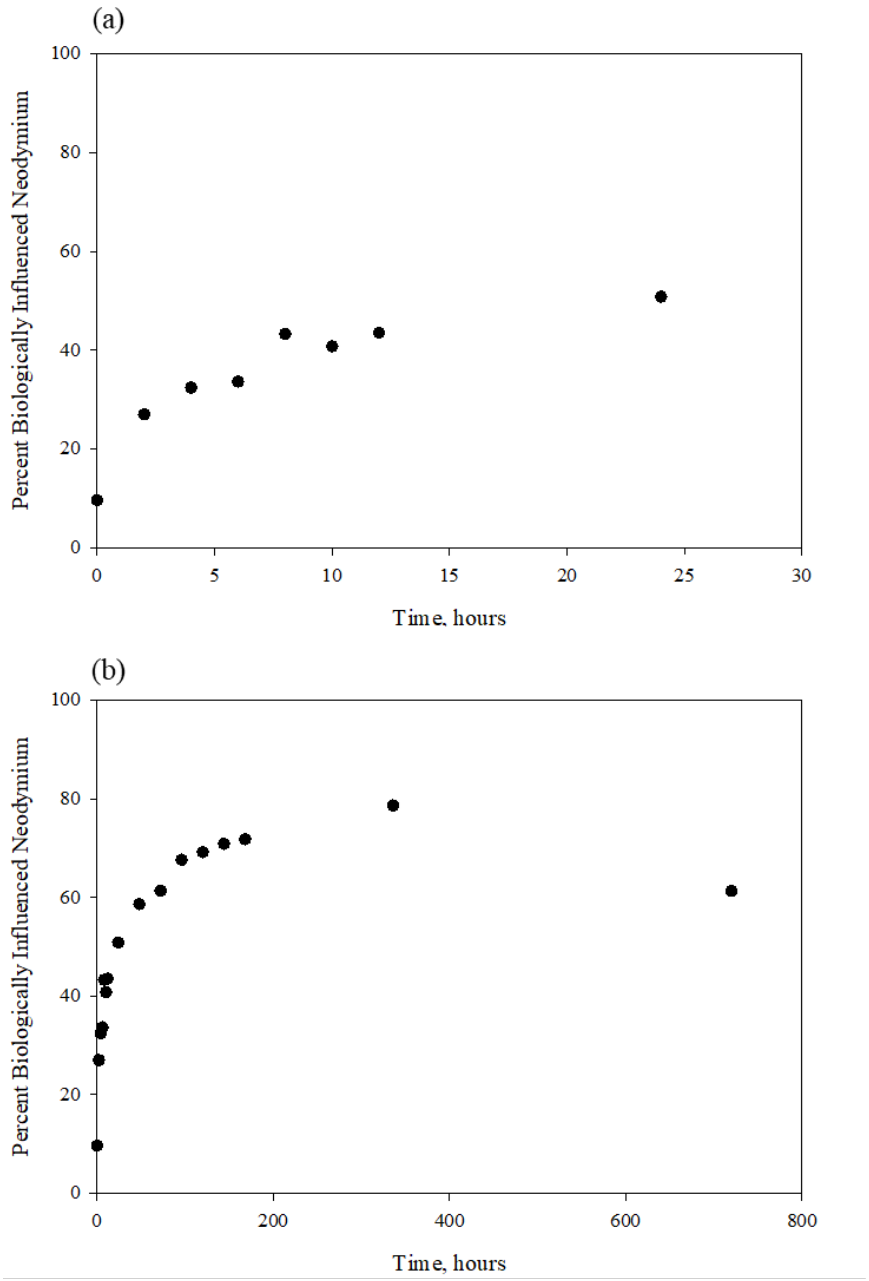


Figure A22. Percent of biologically influenced neodymium in GWB of *Thalassobacillus* sp. spore experiments over a) 24 hours and b) one month.

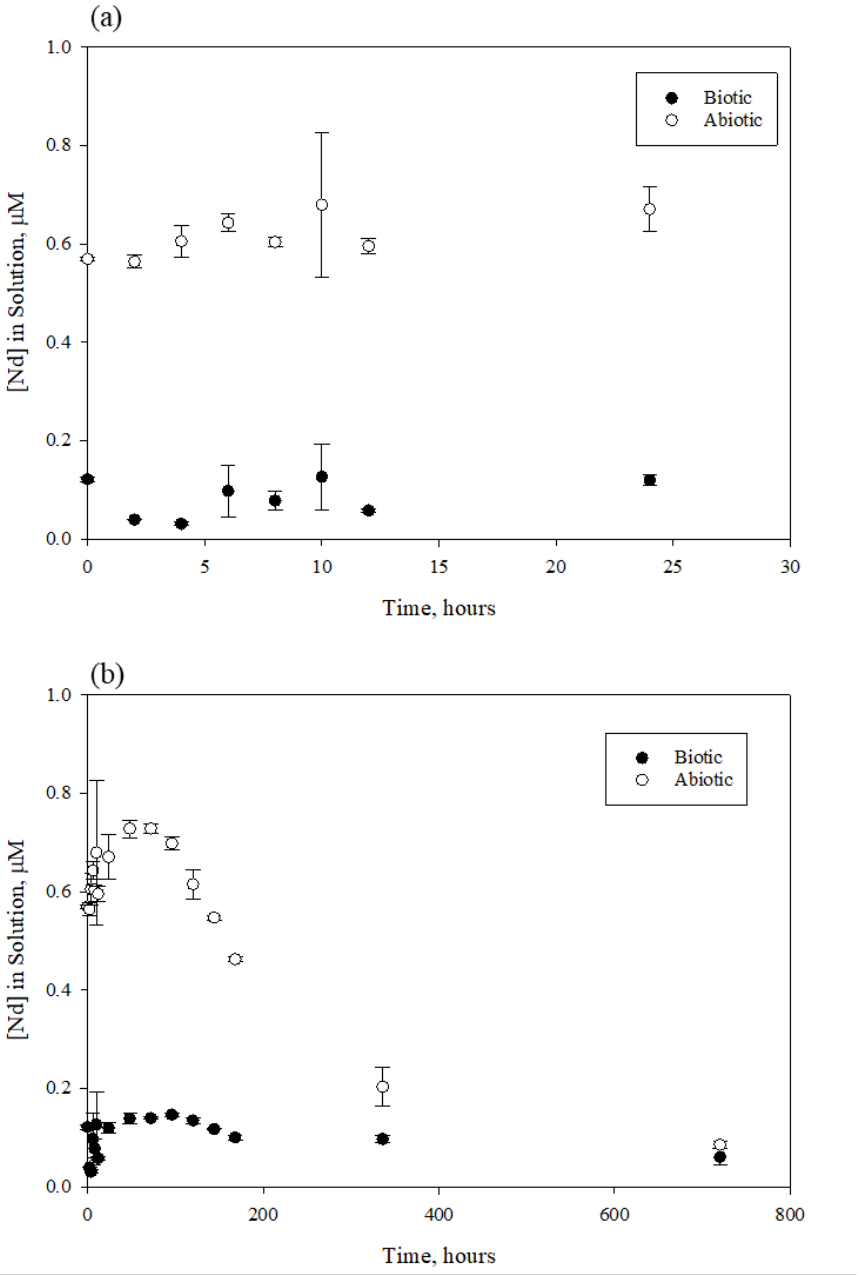


Figure A23. Concentrations of Nd in biotic and abiotic samples from *Thalassobacillus* sp. spore experiments in ERDA over a) 24 hours and b) one month. Error bars represent the deviation from the mean of triplicate measurements.

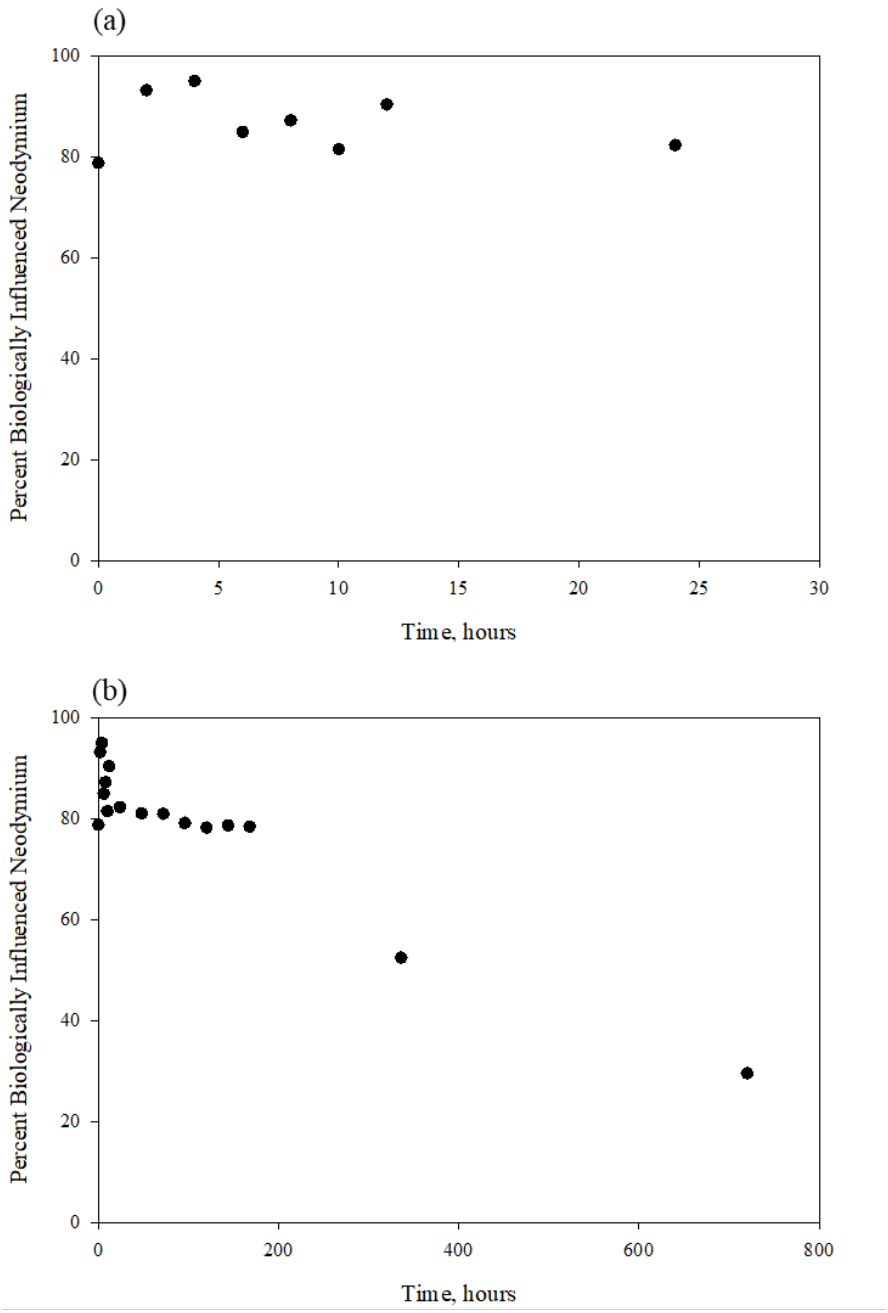


Figure A24. Percent of biologically influenced neodymium in ERDA of *Thalassobacillus* sp. spore experiments over a) 24 hours and b) one month.

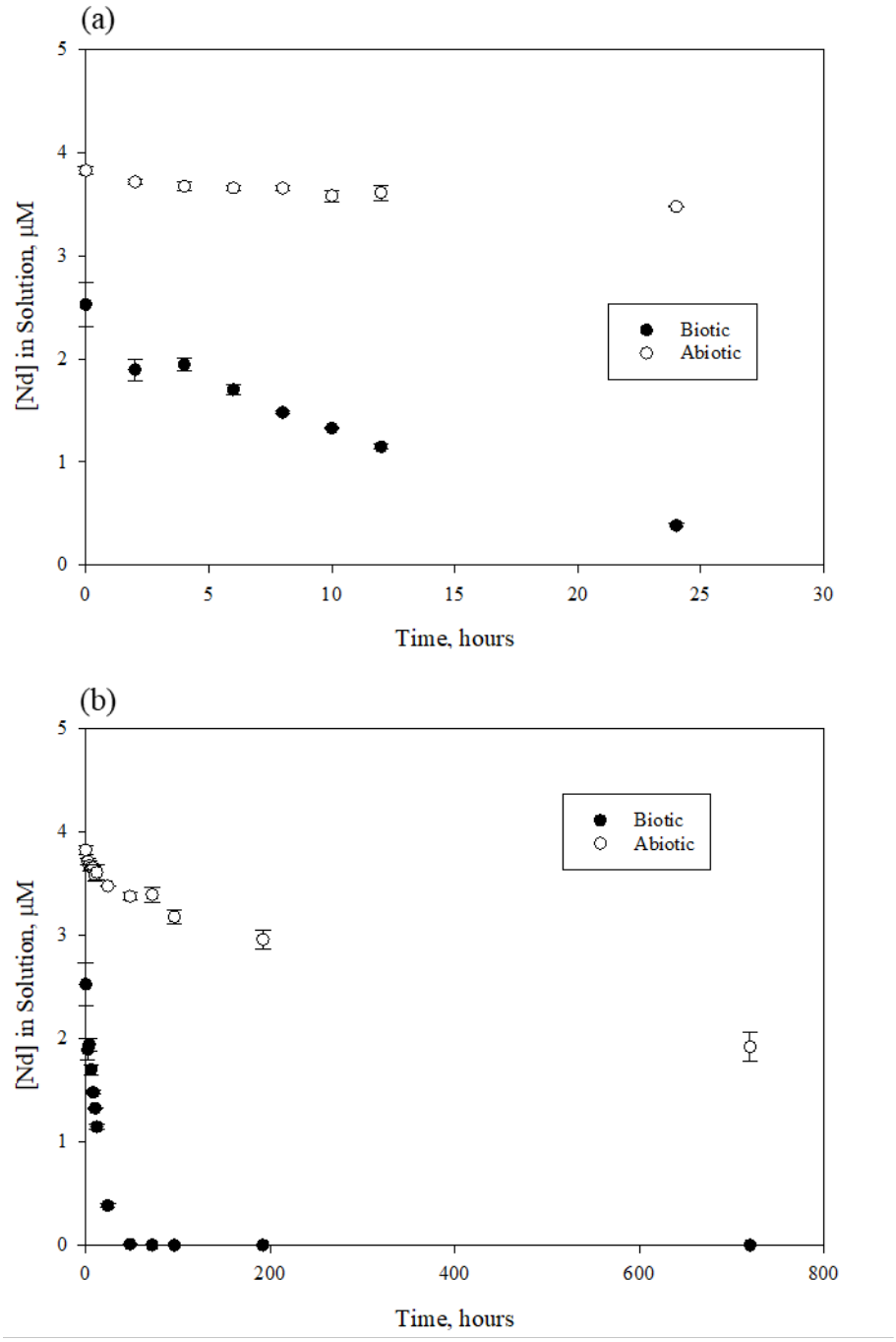


Figure A25. Concentrations of Nd in biotic and abiotic samples from experiments of archaeal isolate PB-1a in 4.3 M NaCl over a) 24 hours and b) one month. Error bars represent the deviation from the mean of triplicate measurements.

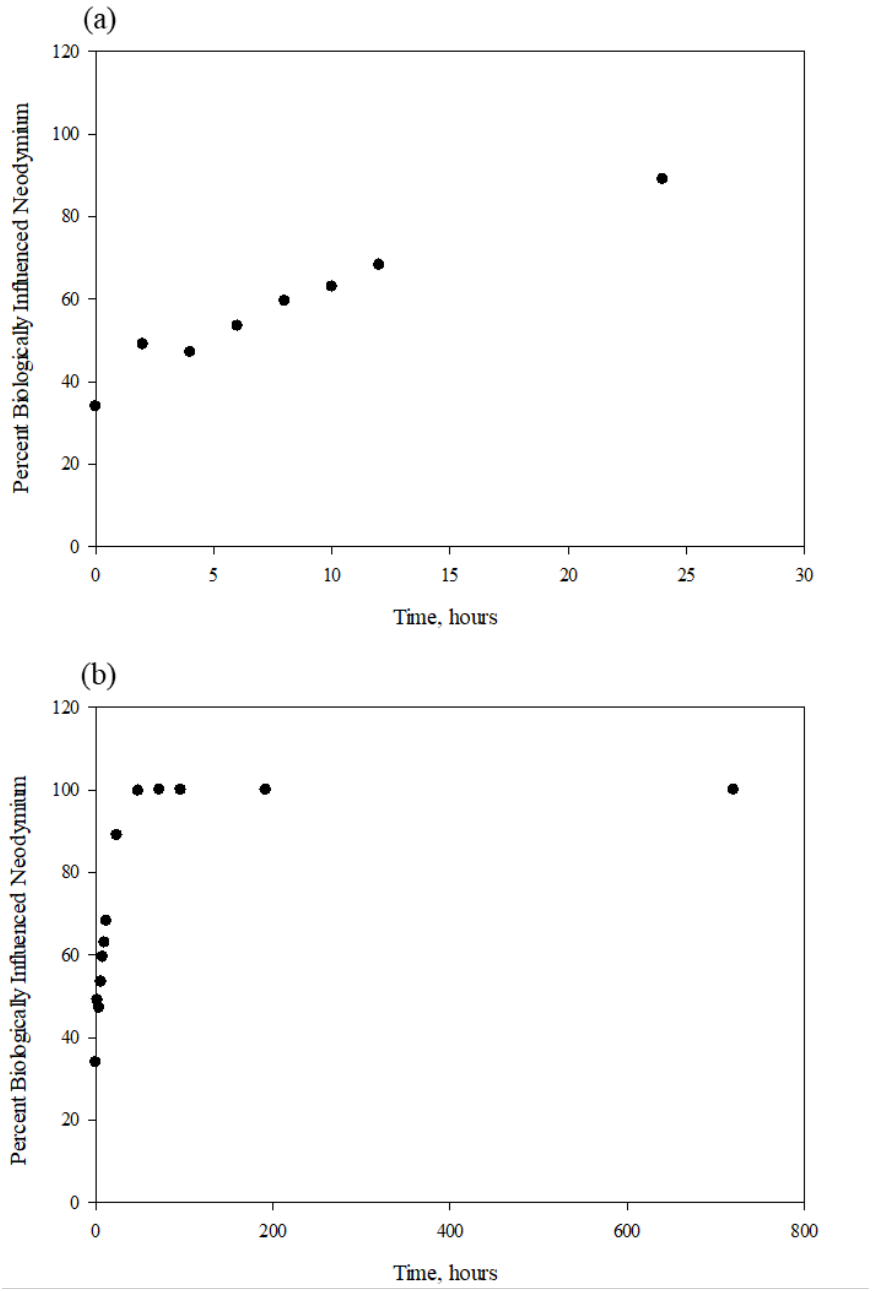


Figure A26. Percent of biologically influenced neodymium in 4.3 M NaCl of experiments with archaeal isolate PB-1a over a) 24 hours and b) one month.

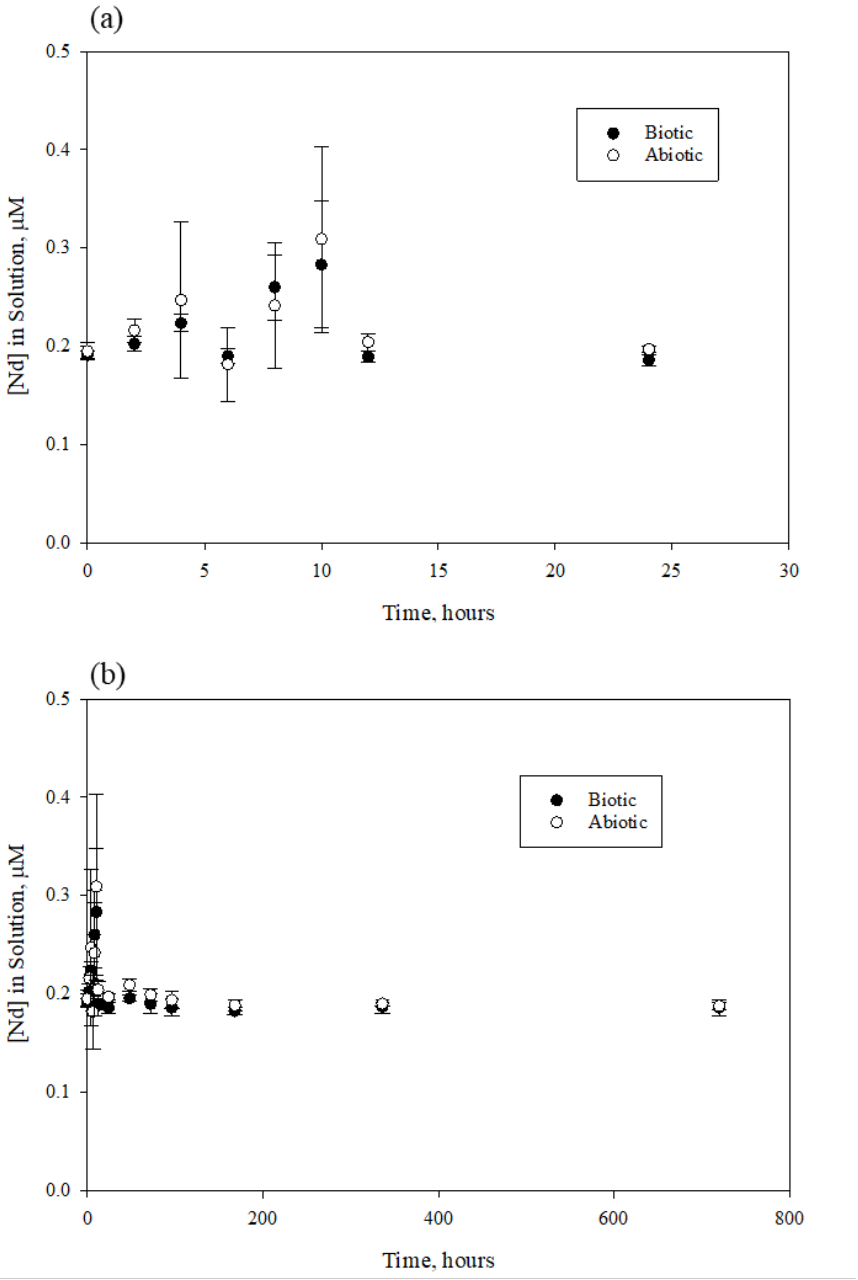


Figure A27. Concentrations of Nd in biotic and abiotic samples from experiments of archaeal isolate PB-1a in GWB over a) 24 hours and b) one month. Error bars represent the deviation from the mean of triplicate measurements.

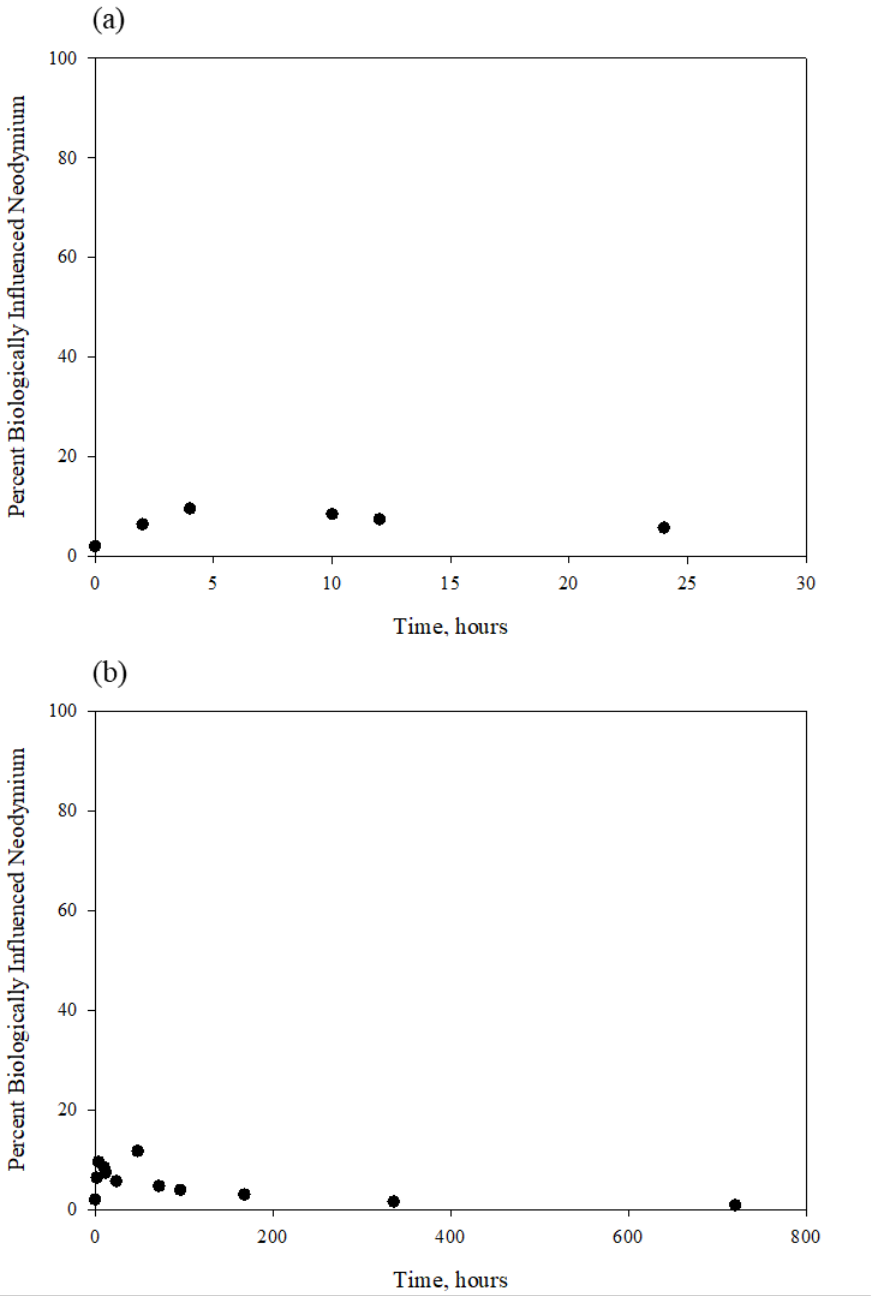


Figure A28. Percent of biologically influenced neodymium in GWB of experiments with archaeal isolate PB-1a over a) 24 hours and b) one month.

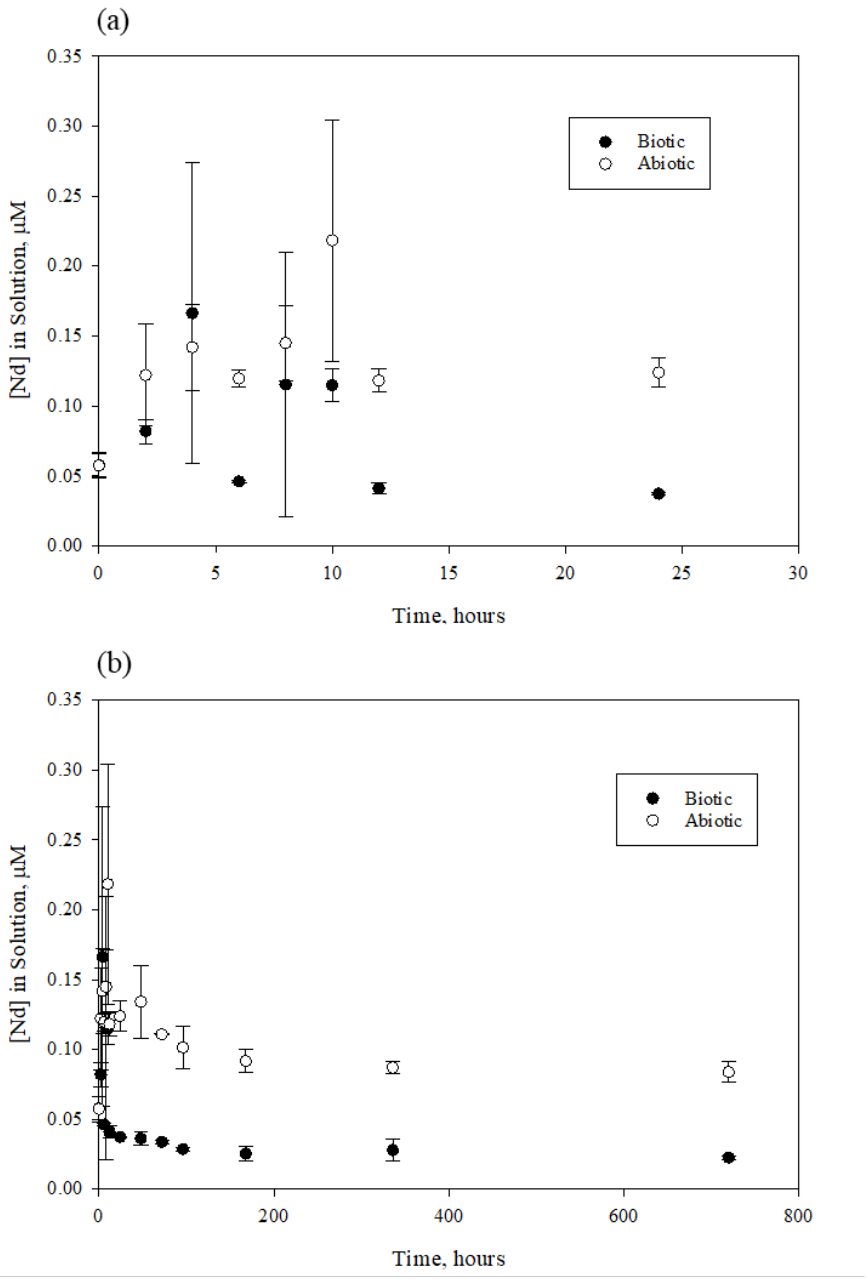


Figure A29. Concentrations of Nd in biotic and abiotic samples from experiments of archaeal isolate PB-1a in ERDA over a) 24 hours and b) one month. Error bars represent the deviation from the mean of triplicate measurements.

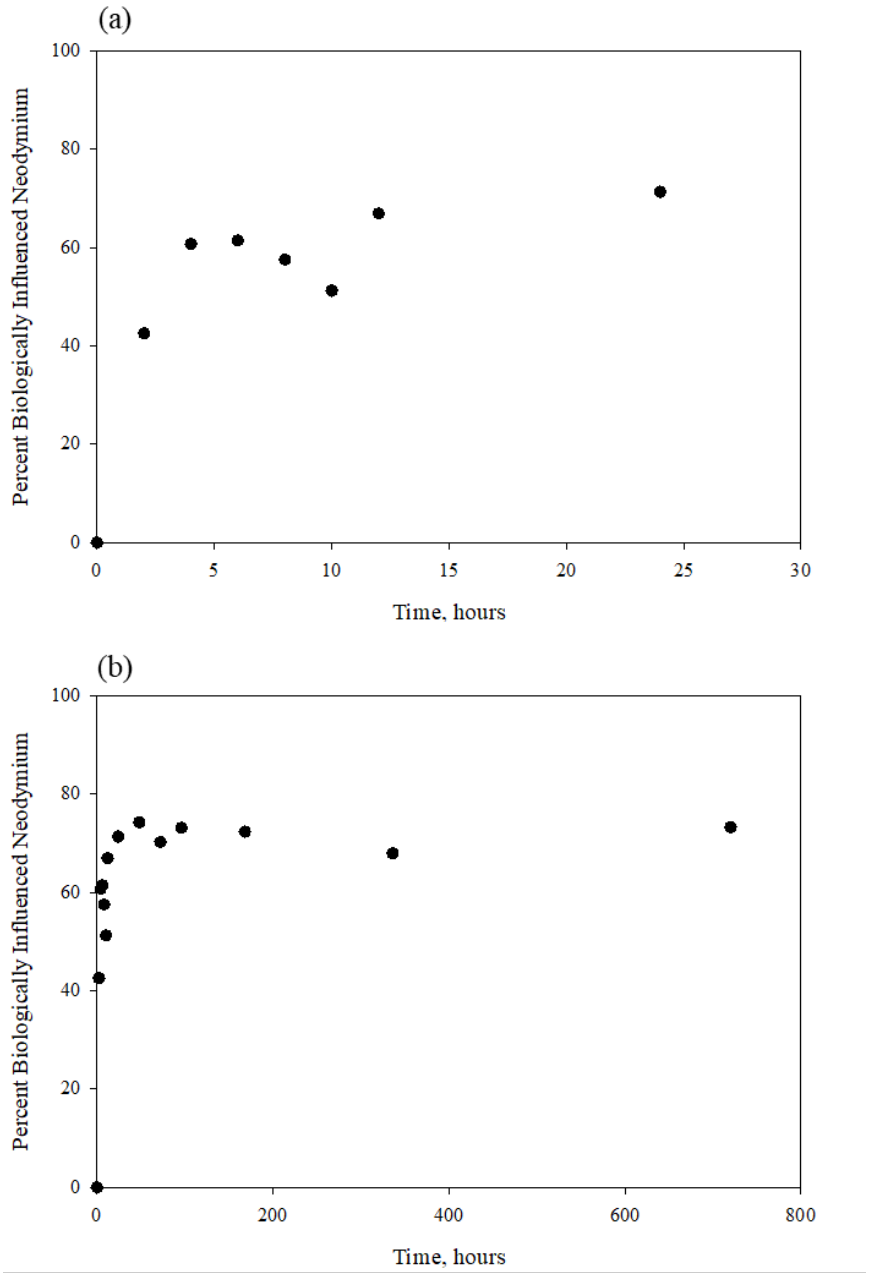


Figure A30. Percent of biologically influenced neodymium in ERDA of experiments with archaeal isolate PB-1a over a) 24 hours and b) one month.

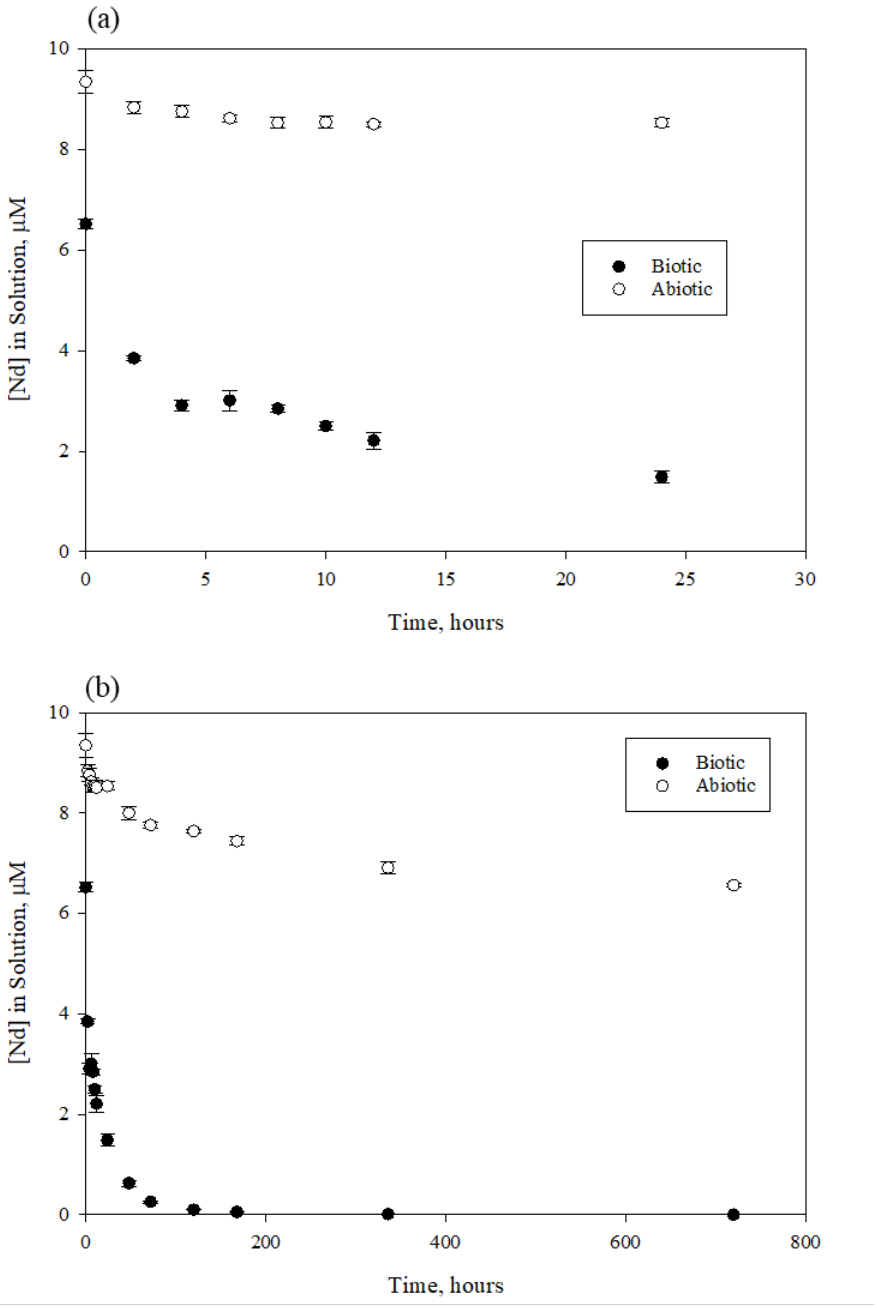


Figure A31. Concentrations of Nd in biotic and abiotic samples from experiments of archaeal isolate Trans-1b in 3.0 M NaCl over a) 24 hours and b) one month. Error bars represent the deviation from the mean of triplicate measurements.

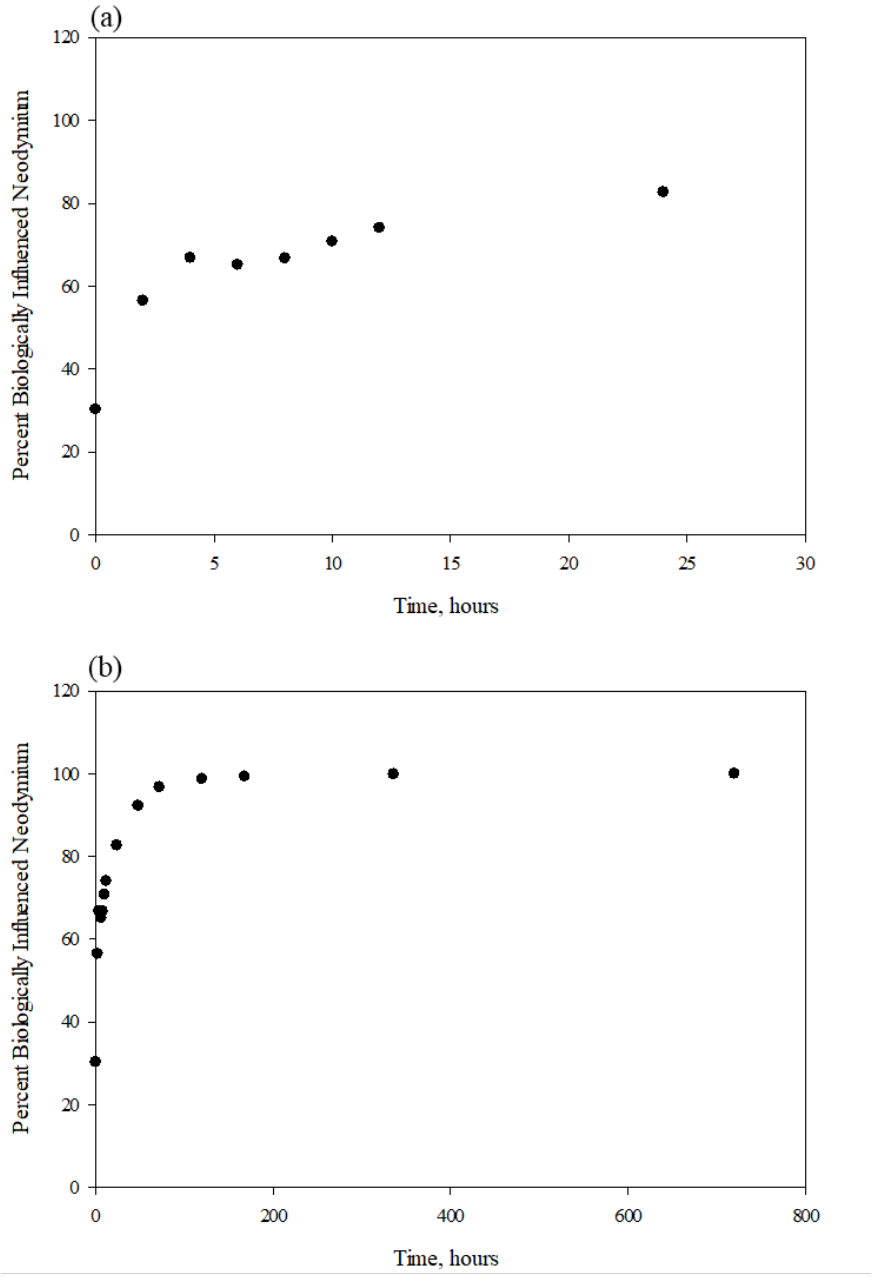


Figure A32. Percent of biologically influenced neodymium in 4.3 M NaCl of experiments with archaeal isolate Trans-1b over a) 24 hours and b) one month.

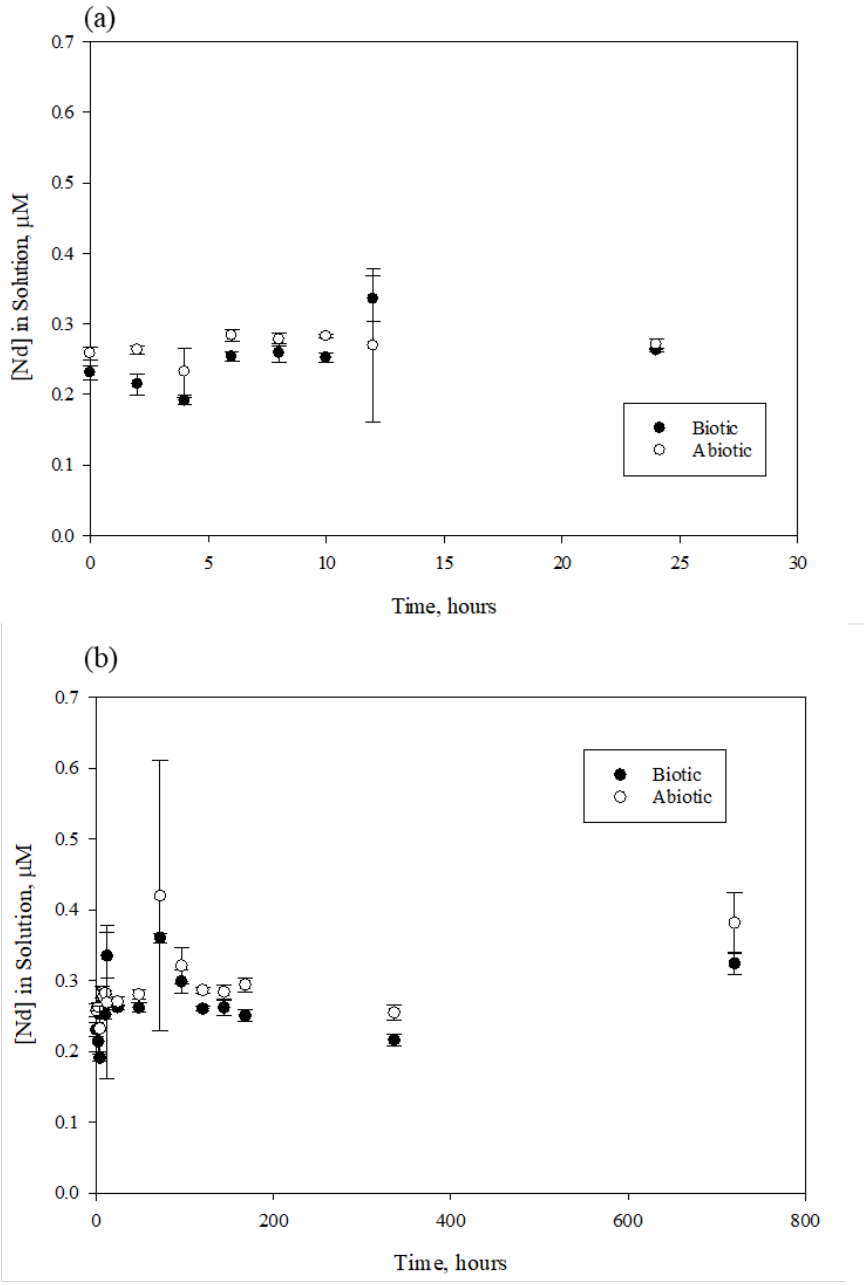


Figure A33. Concentrations of Nd in biotic and abiotic samples from experiments of archaeal isolate Trans-1b in GWB over a) 24 hours and b) one month. Error bars represent the deviation from the mean of triplicate measurements.

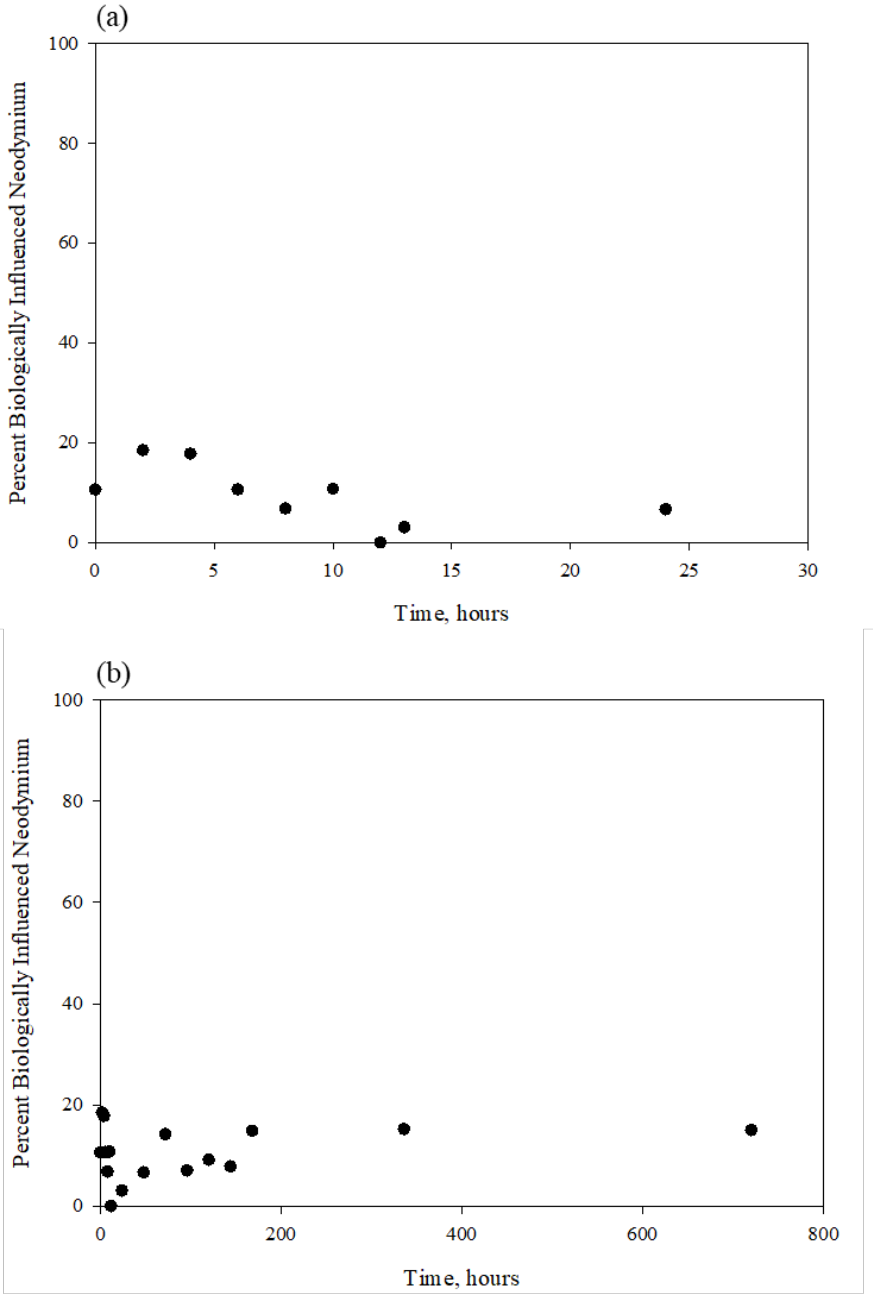


Figure A34. Percent of biologically influenced neodymium in GWB of experiments with archaeal isolate Trans-1b over a) 24 hours and b) one month.

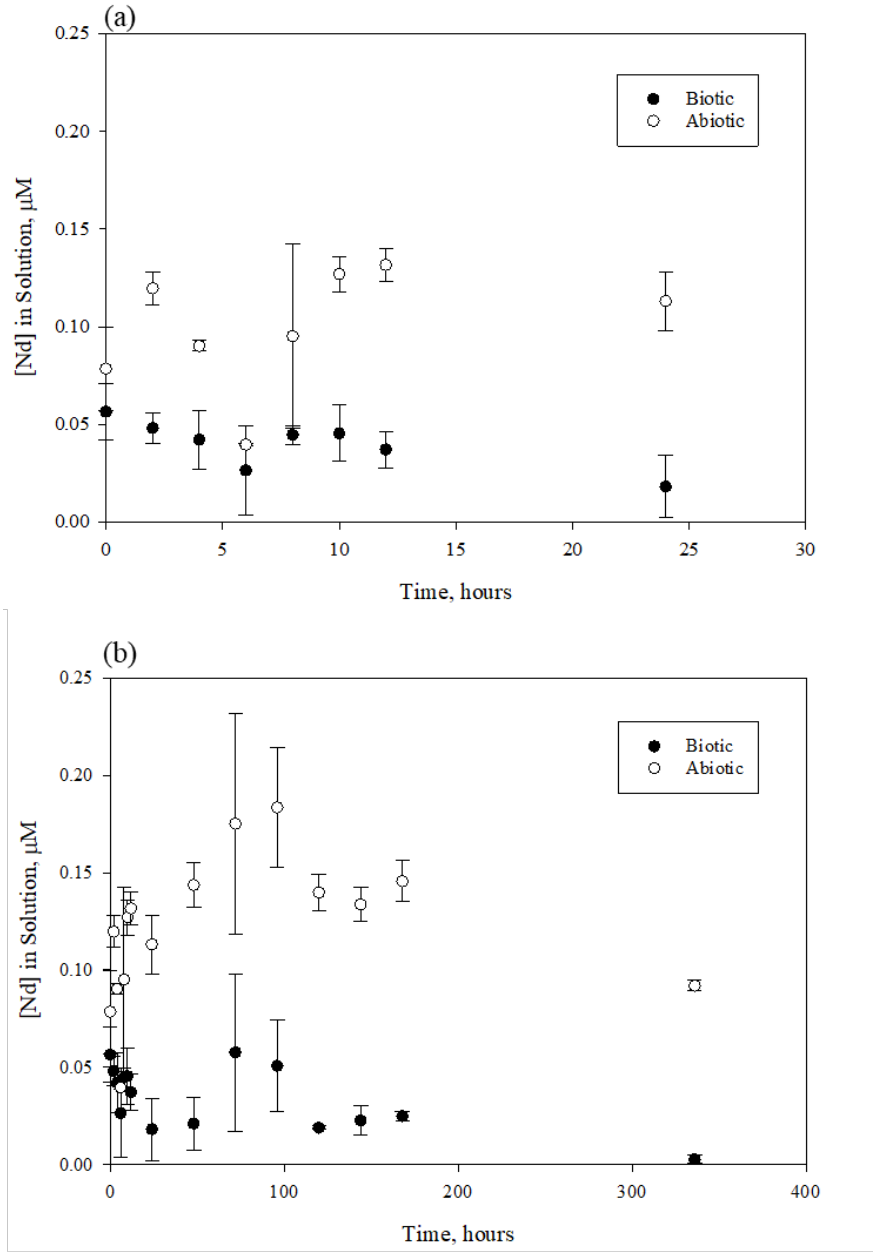


Figure A35. Concentrations of Nd in biotic and abiotic samples from experiments of archaeal isolate Trans-1b in ERDA over a) 24 hours and b) 2 weeks. Error bars represent the deviation from the mean of triplicate measurements.

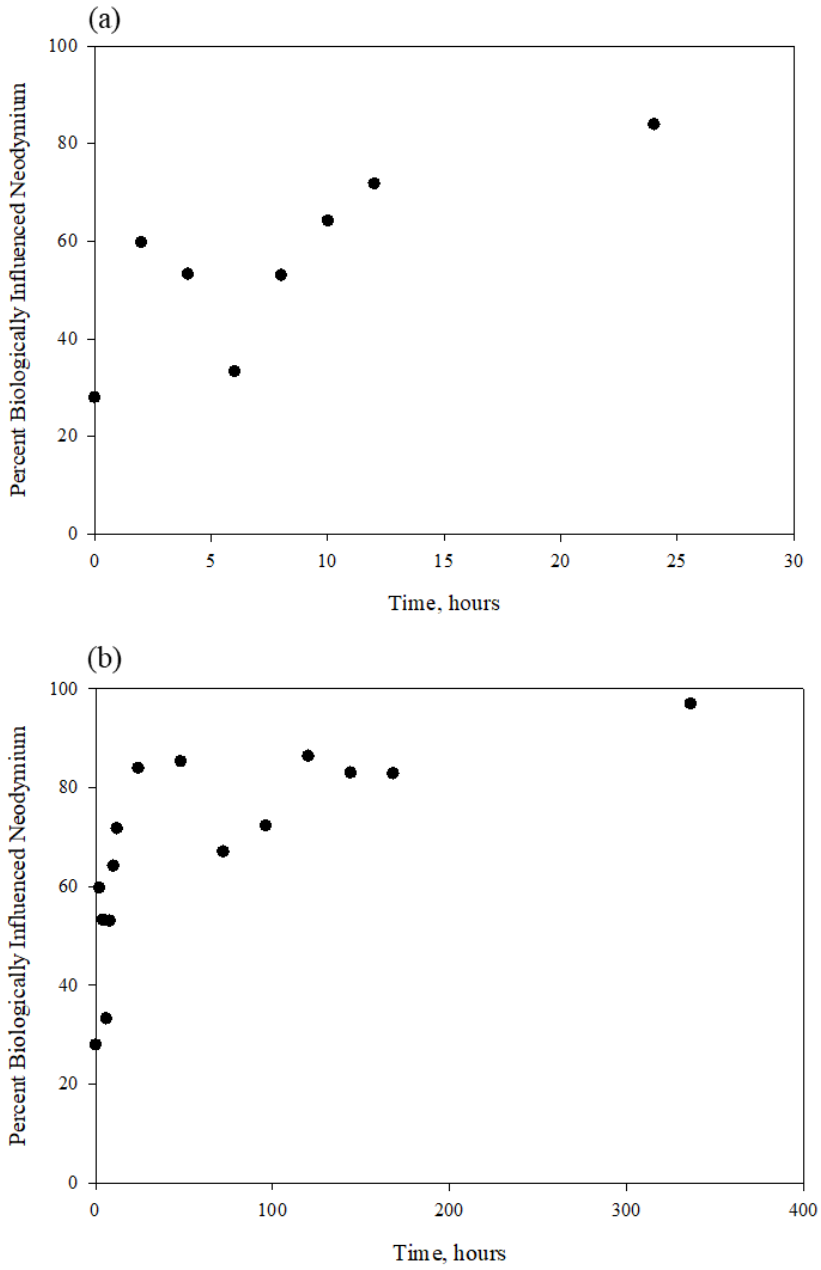


Figure A36. Percent of biologically influenced neodymium in ERDA of experiments with archaeal isolate Trans-1b over a) 24 hours and b) 2 weeks.