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Water Impact Statement

Brine waste associated with ion-exchange technologies comprises a major portion of their economic and environmental impacts. Here, biocatalysts were shown to reduce regulated contaminants perchlorate and nitrate in synthetic and real-world brines. A hybrid ion-exchange/biocatalytic process could prevent the reintroduction of perchlorate and nitrate into the environment and facilitate brine reuse.

Biocatalytic Removal of Perchlorate and Nitrate in Ion-Exchange Waste Brine

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Abstract

Biocatalytic technologies are characterized by targeted, rapid degradation of contaminants over a range of environmentally relevant conditions representative of groundwater, but have not yet been integrated into drinking water treatment processes. This work investigated the potential for a hybrid ion-exchange/biocatalytic process, where biocatalysis is used to treat ion-exchange waste brine, allowing reuse of the brine. The reduction rates and the fate of the regulated anions perchlorate and nitrate were tested in synthetic brines and a real-world waste brine. Biocatalysts were applied as soluble protein fractions from *Azospira oryzae* for perchlorate reduction and *Paracoccus denitrificans* and *Haloferax denitrificans* for nitrate reduction. In synthetic 12% brine, the biocatalysts retained activity, with rates of 32.3 ± 6.1 U (μg Mo)$^{-1}$ for perchlorate (*A. oryzae*) and 16.1 ± 7.1 U (μg Mo)$^{-1}$ for nitrate (*P. denitrificans*). In real-world waste brine, activities were slightly lower (20.3 ± 6.5 U (μg Mo)$^{-1}$ for perchlorate and 14.3 ± 3.8 U (μg Mo)$^{-1}$ for nitrate). The difference in perchlorate reduction was due to higher concentrations of nitrate, bicarbonate, and sulfate in the waste brine. The predominant end products of nitrate reduction were nitrous oxide or dinitrogen gas, depending on the source of the biocatalysts and the salt concentration. These results demonstrate biocatalytic reduction of regulated anions in a real-world waste brine, which could facilitate brine reuse for the regeneration of ion-exchange technologies and prevent reintroduction of these anions and their intermediates into the environment.

Keywords

Azospira oryzae; Paracoccus denitrificans; Haloferax denitrificans; ion-exchange brine; salt sensitivity

1. Introduction

Ion-exchange technologies are effective at removing a range of charged contaminants in drinking water treatment and serve as the best available technology for many inorganic anions, radionuclides, and metalloids.[1] However, regenerating ion-exchange resins produces a contaminated brine waste, which is principally disposed of through dilution and municipal wastewater treatment.[2] The brine and its disposal result in three negative consequences: i) increasing the financial costs of treatment, ii) contributing a significant portion of the environmental impacts, and iii) reintroducing the contaminants into the environment.[3, 4] This work seeks to minimize those consequences by developing a biocatalytic treatment process for ion-exchange waste brines, focusing on the regulated anions perchlorate[5] and nitrate.[1]

Brine regeneration for perchlorate and nitrate contamination has been investigated previously using chemical reduction, chemical catalysts, or whole-cell biological technologies.[3, 6-8] Chemical reduction produces ammonium, which must then be removed.[8] Chemical catalysts rely on hydrogen as the source of the electrons.[7] Whole-cell biological reduction has been tested using a variety of electron donors, including hydrogen[9, 10] and acetic acid.[3] Whether chemical or biological, processes that use hydrogen as an electron donor can minimize the growth of excess biomass since no additional carbon is added to the system. However, this electron donor can be volatile (if supplied as liquid hydrogen), corrosive, and explosive. An acetic acid-based whole-cell process was tested at the pilot scale, where it had effective nitrate and perchlorate reduction in waste brines.[3] While capital costs would be approximately 14.3% higher per 1000 gallons of treated water for whole-cell brine treatment, the operation and maintenance were predicted to yield a significant cost reduction of 29.7% per 1000 gallons of treated water. Whole-cell biological reduction has also been tested with direct contact between the ion-exchange resin and

the bacterial cells, both in the absence and presence of sodium chloride.[11, 12] In these systems, biological degradation of perchlorate without brine resulted in incomplete regeneration of the resin (greater than 40% resin-bound perchlorate remaining after 8 days of incubation).[11, 12] To avoid the effects of biofouling, another study proposed combined brine and resin regeneration by separating the resin and the bacterial cells with a membrane; this study reported better resin capacity than the previous study over six regeneration cycles.[13] In the whole-cell biological regeneration systems, to the best of our knowledge, the fate of the contaminants, especially nitrate, has not been thoroughly evaluated. Furthermore, although these prior reports support the potential benefits of brine treatments, to our knowledge, no such process has been implemented at full-scale.

Recent work has demonstrated the reduction of perchlorate using cell-free enzymes as biocatalysts in drinking water sources[14, 15] and described the required technology improvements that would allow this approach to be competitive financially and environmentally.[16] Compared to chemical catalysts, the biocatalysts have the advantages of performing well under typical groundwater pHs and of having faster reduction rates.[7, 15] Compared to whole-cell processes, the biocatalysts have the advantages of being less sensitive to nitrate, having no observed activity for sulfate, and having no effects from sulfate on perchlorate-reducing activity.[14] The biocatalysts themselves are inert and therefore do not require supplemental nutrients.[14] This inert state reduces the amount of electron donor that is required, because none is going towards biomass production. The lower electron donor concentration also reduces the potential for growth of other microorganisms, which might include pathogens. However, to our knowledge, the response of perchlorate-reducing biocatalysts to salt concentration has not previously been reported.

One focus of this work was to investigate the effects of synthetic and real-world waste brine on the perchlorate-reducing activity of biocatalysts. Since nitrate is also an important contaminant in waste brines, we also sought to develop a biocatalytic approach for nitrate reduction and test its response to brine conditions. The fates of both contaminants were also evaluated. This characterization of the activity and contaminant fate was designed to provide an assessment of the technical feasibility of biocatalytic treatment for ion-exchange waste brines. The long-term motivation of this work includes minimizing contaminant reintroduction into the environment, converting contaminants into innocuous end products, and improving ion-exchange treatment's economic and environmental sustainability.

2. Materials and Methods

2.1 Biocatalyst Preparation, Media, and Chemicals

Laboratory solutions were prepared with Nanopure water (18 MΩ cm) from deionized water (EMD Millipore Milli-Q System, Billerica, MA). Unless otherwise specified, chemicals were purchased from Fisher Scientific (Pittsburgh, PA). Anaerobic solutions were degassed with N_2 with CO_2 for 30 minutes, and headspace was degassed with the same mixture for 5 minutes. The target ratio of N_2 to CO_2 was 80:20. Experiments were performed in triplicate from independent growths (biological replicates) and reported with standard deviation unless noted otherwise. Open anaerobic solutions were handled in an anaerobic glovebox chamber (95% N_2, 5% H_2) and used within an hour.

Biocatalysts were obtained from the perchlorate-reducing *Azospira oryzae* strain PS (ATCC number BAA-33), the nitrate-reducing *Paracoccus denitrificans* (ATCC number 19367), and the marine nitrate-reducing *Haloferax denitrificans* (ATCC 35960). *A. oryzae* was grown anaerobically on perchlorate (7mM), harvested by centrifugation, lysed by sonication, and

centrifuged to separate the soluble protein fraction (SPF), all as described previously.[14, 15] Similar procedures were followed for the other two strains, with the following modifications. For *P. denitrificans*, 11.8 mM sodium nitrate was used for growth instead of perchlorate. *H. denitrificans* was initially grown aerobically in YH medium as previously described.[17] The anaerobic growth media for *H. denitrificans* again contained nitrate instead of perchlorate and was also supplemented with 175.2 g sodium chloride, 1.9 g potassium chloride, 0.1 g calcium chloride dihydrate, and 19.8 g magnesium chloride hexahydrate per L of media. Throughout the preparation of the SPF from *H. denitrificans*, 12% sodium chloride was included in the buffers.

To compare across independent preparations and strains, perchlorate, chlorate, and nitrate-reducing activities were normalized to the molybdenum concentration, an indirect measure of perchlorate reductase and nitrate reductase concentration. Molybdenum concentration was measured using inductively coupled plasma-optical emission spectrometry (ICP-OES) (PerkinElmer Optima 2000DV, Waltham, MA). The impact of increasing the sodium chloride concentration on the solubility of the perchlorate-reducing SPF from *A. oryzae* was determined for a single biological replicate. The SPF was incubated in five sodium chloride concentrations (SPF buffer with 0%, 3%, 6%, 9% and 12% NaCl) for 15 minutes. Samples were then centrifuged at 140,000xg for 60 minutes. The soluble fractions were analyzed for their molybdenum content. Total protein in each SPF was also measured using the Bicinchoninic acid (BCA) assay (Pierce, Rockford, IL).

2.2 Brine Characterization

The waste ion-exchange brine was obtained from a California utility and was characterized before use by previously reported methods.[14, 15] Additional analyses included chemical oxygen demand (COD), ammonium, and anions. COD was determined using a digestion solution (Hach

digestion solution). For high (>0.36 mM) ammonia, samples were analyzed by colorimetric analysis (Hach salicylate kit). Perchlorate, chlorate, nitrate, sulfate, bromate, and iodate were analyzed by ion chromatography (IC) as previously described.[14] Including 1:10 sample dilutions due to the high chloride concentration, detection limits for the anions are listed in Table 1.

2.3 Colorimetric Biocatalytic Assays

Perchlorate- and nitrate-reducing activities were analyzed colorimetrically using methyl viologen as the electron donor[14, 18, 19] in stoppered anaerobic cuvettes with 20 µL of SPF at room temperature. Perchlorate and chlorate was added to a final concentration of 1 mM. Nitrate experiments typically had a final concentration of 10 mM. In tests mimicking the nitrate concentration in the waste brine, 151 mM nitrate was included. Assays were conducted over sodium chloride concentrations ranging from 0 to 12% (w/v) and in waste brine. For reactions testing the effects of other anions on the biocatalyst activity, the anions were included in the synthetic brine at concentrations identical to the waste brine before degassing. All anions were sodium form. Chlorite[15] and nitrite could not be tested in this assay due to background activity caused by their reactivity with methyl viologen.

2.4 Perchlorate-End Product Analysis

While perchlorate was measured using the IC, the fate of perchlorate, as determined by measuring the formation of chloride, could not be tested through a typical mass balance approach. This was due to the high concentration of sodium chloride in brine experiments prevented quantification of chloride. Specifically, for chloride, full reduction of 1 mM perchlorate added to the kinetic reactions would only contribute 0.05% change in the chloride concentration at 12% sodium chloride conditions. Due to the small concentration difference, combined with the sample dilutions required to obtain integrable peaks for chloride, we were

unable to distinguish the small chloride contribution from perchlorate reduction in the IC data. The other end product, oxygen, reacts with the electron donors required for perchlorate reductase activity. Instead, perchlorate and chlorate degradation was monitored based on quantification of perchlorate and chlorate in endpoint assays with IC measurements. These endpoint assays contained 100 µL of SPF, electron donor (nicotinamide adenine dinucleotide (NADH), 250 µM), electron shuttle (phenazine methosulfate (PMS), 100 µM), and 50 µM of sodium perchlorate in 100 mL 50 mM MOPS buffer, pH 7.0. or 100 mL of waste brine in 160 mL stoppered serum bottles. Assays were incubated at room temperature (approximately 21.7C) overnight.[15, 20]. After incubation, 3 mL samples were taken and passed through 0.22 µm PES syringe filters for IC analysis. Controls containing perchlorate and chlorate without the SPF were included.

The last step in the perchlorate-reducing pathway, chlorite decomposition, was directly tested in experiments where chlorite was supplied and dissolved oxygen was measured. Dissolved oxygen was measured at room temperature using a DO probe (08005MD, Thermo Fisher Scientific, Waltham, MA), and the probe was calibrated each time with air saturated DI water. Twenty µL of SPF was added to 10mL of 50mM Tris-Cl^- buffer (pH 7.5) in a 25mL beaker with 0 to 12% sodium chloride, or to 10mL of waste brine, with constant stirring. Sodium chlorite was added to a final concentration of 0.18 mM to initiate the reaction. The experiment was monitored until the oxygen values leveled off. A salt correction factor based on temperature and barometric pressure was used to account for the salinity effects on the probe[21] according to manufacturer's instructions. No oxygen formation was observed in buffer-only, buffer plus SPF, or buffer plus sodium chlorite controls. The amount of oxygen formed was compared stoichiometrically with the amount of chlorite added to the reaction. Oxygen formation rates are reported as mg O_2 per second per liter of reaction.

168 **2.5 Nitrate-End Product Analysis**

169 To determine the fate of nitrate, additional endpoint assays were conducted. In these assays,
170 100 µL of *A. oryzae, P. denitrificans,* or *H. denitrificans* SPF was incubated as described above,
171 except that isotopically-enriched nitrate (98% $Na^{15}NO_3$, Sigma Aldrich, St Louis, MO, 25µM)
172 was included instead of perchlorate.[20] Sodium chloride concentrations were varied from 0% to
173 12%. Assays were incubated overnight at room temperature (approximately 21.7 °C). Controls
174 using sodium nitrate and sodium nitrite with no SPF were conducted.

175 For ammonium analysis, ten mL of aqueous samples were filtered through 0.22 µm acetate
176 filters and stored at -80°C until analysis. Ammonium was quantified using the standard phenate
177 method.[22]

178 For nitrous oxide analysis, fifteen mL of gas from the headspace was transferred to a
179 vacuumed (-20 in. Hg) 10 mL vial for analysis. Nitrous oxide was measured using gas
180 chromatography (GC, Shimadzu GC-2014 with Auto Sampler AOC 5000 Plus) with electron
181 capture detector, with a detection limit of 0.1 ppmv.

182 To quantify $^{15}N_2$, one mL of gas from the assay bottle headspace was transferred directly to
183 the instrument for gas chromatography-isotope ratio mass spectroscopy (GC-IRMS, Isoprime
184 100 and Isoprime Trace Gas Analyzer, Isoprime Ltd, Cheadle Hulme, UK) analysis. The setup
185 included modifications to the Isoprime Trace Gas Analyzer, and the instrument drift was
186 accounted for by applying k-factor corrections as previously reported.[23] Instrument precision was
187 2×10^{-6} % ^{15}N. The measured ratio of mass 30 was used to estimate the amount of dinitrogen
188 formed from nitrate reduction by multiplying the ratio and the amount of total nitrogen in the
189 headspace (80% at 5psi).[23]

2.6 Statistical Analysis

Statistical analysis was performed in OriginPro 2017. A standard linear curve fit was used for anions except chlorate. *A. oryzae* SPF activity with chlorate was fit using a non-linear exponential fit. Curve fits are reported with coefficient of determination (R^2). Normality was tested using Shapiro-Wilks test. A Kruskal-Wallis test was initially performed to determine significant differences in data groups. Subsequent differences were tested using the Mann Whitney U Test. Significance was determined at an alpha less than 0.05.

3. Results

3.1 Characterization of Brine

Waste brine was obtained from a full-scale drinking water treatment plant that uses ion exchange to remove nitrate. The sodium chloride concentration in the waste brine was similar to a synthetic 12% brine. The brine had background concentrations of nitrate (151 mM) but no detectable levels of perchlorate (Table 1). Other anions in the perchlorate and nitrate-reducing pathways were not detected. Sulfate and bicarbonate levels were 25.5 mM and 122 mM, higher than concentrations previously tested for the perchlorate-reducing biocatalysts.[14]

3.2 Characterization of SPF

As in previous reports, biocatalysts were used as SPFs; they were not purified.[14] Throughout this work, the activities of the SPF were normalized to molybdenum, as the subunit A of perchlorate reductase and subunit NarG of nitrate reductase each contain one molecule of molybdenum.[18, 24] SPFs produced in this work contained an average of 17.6 ± 0.4 mg protein mL^{-1} and 383 ± 47 μg Mo L^{-1} for *A.oryzae* and 11.5 ± 1.0 mg protein mL^{-1} and 418 ± 17 μg Mo L^{-1} for *P. denitrificans*. For the stable isotope experiments, data from a single SPF from

Haloferax denitrificans is reported. This SPF had a protein concentration of 14.1 ± 0.8 mg protein mL^{-1}.

3.3 Biocatalytic Activity in Synthetic and Waste Brine

Due to the structural similarity between perchlorate reductase and nitrate reductase,[25] SPFs from perchlorate-reducing and denitrifying bacteria were both tested with perchlorate and with nitrate. Considering perchlorate-reducing activity first, no activity was detected with *P. denitrificans* SPF, regardless of the sodium chloride concentration. The *A. oryzae* SPF had the highest perchlorate-reducing activity in the absence of sodium chloride, 146.7±21.8 U $(\mu g\ Mo)^{-1}$, which was consistent with previously reported values of 162.5±8.4 U $(\mu g\ Mo)^{-1}$.[15]. With increasing salt concentration, the activity decreased linearly, with an R^2 fit value of 0.999 (Figure 1). However, even at 12% sodium chloride, the *A. oryzae* SPF retained a perchlorate-reducing activity of 32.3±6.1 U $(\mu g\ Mo)^{-1}$. The *A. oryzae* SPF had higher activity with chlorate compared to perchlorate (Figure 1). Chlorate reduction decreased exponentially (R^2=0.983), suggesting the activity is more sensitive to the initial increase in sodium chloride concentrations. However, chlorate-reducing activity maintained a factor of at least 1.8 times greater activity than perchlorate-reducing activity across all tested sodium chloride concentrations and was sustained even at 12% sodium chloride. The observed inhibition on perchlorate-reducing activity was not specific to sodium chloride for perchlorate reduction. In a mixture of 6% sodium chloride and 3.7% sodium sulfate, the biocatalytic activity decreased to 42.8 ± 3.6 U $(\mu g\ Mo)^{-1}$. This activity is slightly less than the observed activity at 9% sodium chloride.

Nitrate reduction decreased linearly with increasing sodium chloride concentration for both *A. oryzae* and *P. denitrificans* SPFs, but again was sustained even at 12% sodium chloride (Figure 1). In buffered solution without sodium chloride added, the *A. oryzae* SPF had a nitrate-

reducing activity of 105.6±18.7 U (μg Mo)$^{-1}$, and the activity decreased linearly with an R^2 fit value of 0.998, to 28.7±3.2 U (μg Mo)$^{-1}$ in 12% sodium chloride. With the *P. denitrificans* SPF, nitrate-reducing activity started at 188.9±8.1 U (μg Mo)$^{-1}$for the buffered solution, similar to a previously published value for pure nitrate reductase of 285 U (μg Mo)$^{-1}$.[26] The activity decreased linearly with increasing sodium chloride (R^2 fit of 0.991),with a nitrate-reducing activity of 16.1±7.1 U (μg Mo)$^{-1}$ in buffered 12% sodium chloride solution. For SPF from *A. oryzae*, incubation under high sodium chloride concentrations did not cause a change in the concentration of molybdenum in solution (data not shown), which suggests that the biocatalysts remained soluble.

Building on these promising results, perchlorate reduction was then tested using a real-world waste brine spiked with 1 mM perchlorate (Figure 2). The *A. oryzae* SPF had a 37.1% decrease in activity in the waste brine (20.3±6.5 U (μg Mo)$^{-1}$ in the waste brine versus 32.3±6.1 U (μg Mo)$^{-1}$ in the synthetic brine). Interpretation of this activity was however complicated by the presence of nitrate in the waste brine and the activity of *A. oryzae* SPF with nitrate; without spiking perchlorate, this SPF already had an activity of 17.4±2.7 U (μg Mo)$^{-1}$.

To better understand the effects of other anions present in the waste brine, the composition of the synthetic brine was systematically varied with the *A. oryzae* SPF (Figure 2). Bicarbonate caused a slight but not statistically significant decrease in perchlorate-reducing activity. Sulfate also caused a slight decrease; in this case the decrease was statistically significant. In a synthetic brine with no sulfate and bicarbonate, but where nitrate concentrations mimicked those in the waste brine (151mM), the perchlorate-reducing activity decreased to 11.5±1.3 U (μg Mo)$^{-1}$. A synthetic brine containing similar bicarbonate, sulfate, and nitrate concentrations to the waste brine showed good agreement with the real-world brine experiments. For nitrate reduction, this

combination had 16.9±0.6 U (μg Mo)$^{-1}$, as compared to 17.4±2.7 U (μg Mo)$^{-1}$ in the waste brine (P = 0.28). For perchlorate reduction, the activity was 17.4 ± 1.0 U (μg Mo)$^{-1}$, compared to 20.3 ± 6.5 U (μg Mo)$^{-1}$ in the waste brine (P = 0.33).

The nitrate-reducing SPF from *P. denitrificans* was also tested with the waste brine, which contained 151 mM nitrate (Figure 2). Compared to the simplified synthetic system with 10 mM nitrate, a slight decrease in activity was observed in the waste brine, to 14.3±3.8 U (μg Mo)$^{-1}$. However, increasing the nitrate concentration in a synthetic brine to 151mM, to mimic the waste brine, resulted in an activity of 13.9±3.6 U (μg Mo)$^{-1}$, not significantly different from the waste brine (P = 0.67).

3.4 Perchlorate Fate

Technical constraints prevented a direct analysis of all substrates, intermediates, and products in a single experiment, as detailed in the methods section. Since activity for perchlorate and chlorate was demonstrated (Figure 1) and no perchlorate was detected in the end-point assays at high salt concentrations, experiments focused on testing the activity of chlorite dismutase at varying salt concentrations, measuring oxygen formation from chlorite. The chlorite dismutase activity was more robust then the overall perchlorate activity in the methyl viologen assay, as no statistically significant decrease in activity was detected up to 9% sodium chloride (P = 0.09, 0.55 ± 0.04 mg O_2 L^{-1} s^{-1}) (Figure 3). At the highest sodium chloride concentration, the average chlorite dismutase activity had a statistically significant decrease to 0.51 ± 0.05 mg O_2 L^{-1} s^{-1} at 12% sodium chloride. The average activity of chlorite dismutase in the waste brine was slightly lower than the 12% sodium chloride experiment, at 0.44 ± 0.16 mg O_2 L^{-1} s^{-1}.

The total amount of oxygen formed was tracked, and this mass corresponded well with the mass of chlorite added to the system. Greater than 90% of the expected oxygen was measured for

reactions containing up to 6% sodium chloride. At higher sodium chloride concentrations, the mass of oxygen captured was 89.0% for 9% sodium chloride, 86.3% for 12% sodium chloride, and 69.0% for waste brine. The decrease in the mass balance closure could be accounted for by decreased activity under the latter conditions, which allowed more time for oxygen to diffuse out of the open system.

3.5 Nitrate Fate

The fate of nitrate was tracked by measuring potential products after biocatalytic treatment. Nitrous oxide and dinitrogen were measured as gaseous end products, using stable isotope analysis for the dinitrogen, and ammonium as an aqueous end product. Mass balance calculations suggest there was no accumulation of nitrite or nitric oxide in the synthetic brine system. The stable isotope experiments were conducted in synthetic, buffered systems, and results are based on a single biological replicate with duplicate analytical replicates, unless accompanied by a standard deviation. In control experiments, abiotic conversion of nitrite was observed via direct reaction with the electron shuttle PMS or electron donor NADH. The predominant product of this chemical reaction was ammonium, comprising 75.5% of the total N mass balance. The formation of nitrous oxide was also observed but constituted less than 1% of the total N mass.

The *A. oryzae* SPF yielded nitrous oxide as the dominant product, with 85.5% of the nitrogen recovered as nitrous oxide in 0% sodium chloride and 96.4% in 12% sodium chloride. In both conditions, no $^{15}N_2$ or ammonium was detected from the reaction. These results suggest that while these perchlorate-reducing biocatalysts have activity for nitrate, the *A. oryzae* SPF is unable to reduce nitrous oxide into dinitrogen.

In contrast, the *P. denitrificans* SPF was able to completely reduce the labeled nitrate into dinitrogen at 0% sodium chloride. Under these conditions, no nitrous oxide was detected, and

304 99.4% ± 4.0% dinitrogen was recovered. However, sodium chloride concentrations of 3% or
305 more prevented dinitrogen production; instead, nitrous oxide was formed. Recoveries of nitrous
306 oxide ranged from 83.3% to 99.0% for sodium chloride concentrations from 3% to 12%. Stable
307 isotope detection of dinitrogen gas was not possible with the waste brine, because of its high
308 nitrate concentration, but nitrous oxide was detected after waste brine experiments with SPF
309 from both *A. oryzae* and *P. denitrificans*.
310 As a preliminary attempt to achieve complete reduction of nitrate at elevated brine
311 concentrations, SPF from a salt tolerant denitrifier, *H. denitrificans,* was also tested in stable
312 isotope experiments. The *H. denitrificans* SPF performed worse than *P. denitrificans* at 0%
313 sodium chloride, with only 4.6% of the original nitrate reduced to dinitrogen. However, the *H.*
314 *denitrificans* SPF was the only one to show any dinitrogen formation in 12% sodium chloride
315 (1.4%). The remaining nitrogen was in nitrous oxide, ammonium, and nitrate. Adding both *P.*
316 *denitrificans* and *H. denitrificans* SPF simultaneously (100 μL each) increased the total gaseous
317 nitrogen to 84.3% of the total mass. The dinitrogen yield increased to 25.1% and nitrous oxide
318 comprised 59.2% of the total nitrogen content in 12% sodium chloride. For the combined SPFs,
319 the formation of ammonium was below detection.

320 ## 4. Discussion

321 This work demonstrates biocatalytic reduction of the contaminants perchlorate and nitrate in
322 concentrated synthetic and real-world waste brines containing up to 12% sodium chloride. To
323 our knowledge, it is the first report on the impact of sodium chloride concentration on these
324 enzymes, as well as the first report of biocatalytic nitrate reduction. Differences in the activity
325 for a real-world waste brine compared to synthetic, buffered, laboratory brine were due to the
326 elevated concentrations of bicarbonate, sulfate, and nitrate in the waste brine. Perchlorate was

completely degraded to innocuous end products: chloride and oxygen, while the end products from the reduction of nitrate depended on the source of the biocatalysts and the reaction conditions.

At sodium chloride concentrations relevant to brines used in ion-exchange regeneration, the activities of the enzymes decreased, but substantial activity was retained even at 12% sodium chloride. One possible mechanism for the decrease in perchlorate-reducing activity is product inhibition, since chloride is a product of perchlorate reduction. In the current work, however, the chlorite dismutase in the *A. oryzae* SPF had no apparent inhibition at concentrations up to 9% sodium chloride. Studies on purified enzymes from other organisms have given conflicting results, suggesting that this property varies among isozymes.[27 28] Furthermore, in the current work, the impact of anions on the biocatalytic activity was not unique to perchlorate reduction or to sodium chloride, suggesting a more general mechanism of inhibition. While anion and cation effects from the Hofmeister series may result in the biocatalysts salting out of solution, this explanation was not supported by molybdenum analysis. This would effectively decrease the biocatalysts available for perchlorate and nitrate reduction.[29] Ions can also play a role in the stabilization of particular biocatalysts, as previously shown for purified chlorite dismutase.[28]

Compared to other approaches for perchlorate degradation, the biocatalysts have advantages in effective range, efficient use of the electron donor, and/or reaction rates. In whole-cell systems, some perchlorate-reducing strains were inhibited with the addition of only 2.5 or 5% sodium chloride.[30] A salt-tolerant culture was able to reduce nitrate and perchlorate in real-world 6% waste brine, when amended with magnesium, calcium, and potassium, but was apparently not tested at higher concentrations.[3, 31] In another system, perchlorate and nitrate were reduced in 10% sodium chloride but required acclimation, excess acetate addition, and a 15 hour empty bed

contact time to achieve complete reduction.[32] In contrast, the biocatalysts used here showed immediate activity even at 12% sodium chloride. In our assays, the amount of electron donor required to achieve perchlorate and nitrate reduction had a 4 molar excess ratio versus 10 for whole-cell biological reduction.[32] Unfortunately, the units used in previous publications on whole-cell processes do not permit a direct comparison of the reaction rates with biocatalysis. Both the chemical reduction and existing chemical catalysts are most effective at acidic pH, while the biocatalysts function well under a typical range of pHs for water treatment. Compared to bi-metallic chemical catalysts,[7] the biocatalysts have reaction rates that are three or four orders-of-magnitude faster for perchlorate and nitrate, respectively, when both activities are normalized to the relevant metal content.

The fate of the contaminants is another important consideration. The complete perchlorate reduction pathway was functional even at 12% sodium chloride, and its end products of oxygen and chloride are innocuous. The fate of nitrate in biocatalytic reduction is more complicated, due to its longer pathway and the confounding effect of abiotic ammonium formation between nitrite and the electron donor or shuttle. In brines, the SPF from *A. oryzae* reduced nitrate into nitrous oxide. The reduction of nitrate could have been catalyzed either by perchlorate reductase[25] or by a putative nitrate reductase encoded in the *A. oryzae* genome.[33] There are also putative nitrite and nitric oxide reductases in its genome, which could account for the subsequent conversion to nitrous oxide. *P. denitrificans* has the full denitrifying pathway, and its SPF produced dinitrogen when sodium chloride was not added. However, in brines, the *P. denitrificans* SPF produced nitrous oxide rather than dinitrogen gas, suggesting that the nitrous oxide reductase may be sensitive to the salt concentration. Ammonium formation occurred only with the *H. denitrificans* SPF alone. Based on control experiments, we attribute this to abiotic formation from nitrite and

suggest that biological nitrite reduction may be slower in this organism. Interestingly, although the *H. denitrificans* SPF on its own converted only a small fraction of the nitrate to dinitrogen gas, when the two denitrifying SPFs were added together, approximately a quarter of the nitrate was completely reduced even at 12% sodium chloride. For comparison, chemical reduction produces ammonium, thus requiring an additional treatment process to remove the ammonium.[8] Chemical catalytic reduction converts 30% of the nitrate mass balance to ammonium and 3% to nitrite;[4] both are undesirable end products. To our knowledge, no end product analysis has been reported for whole-cell biological reduction of nitrate in waste brine.

These results suggest that the end products of biocatalytic nitrate reduction could be controlled by the selection of biocatalysts and reactor design. With the large diversity of nitrate-reducing organisms, there may exist biocatalysts better suited for high nitrate concentrations and salinity, and genetic engineering techniques provide additional options for improving the biocatalysts. To decrease production of nitrous oxide, optimization of nitrous oxide reductase would be recommended. Designing a reactor with minimal head space would also help retain nitrous oxide in the aqueous phase, allowing more time for its reduction. Alternatively, the process could be designed for recovery of nitrous oxide. Nitrous oxide has been proposed to enhance the power output of biogas in wastewater treatment,[34] but it is unlikely that water treatment facilities would have access to biogas. However, with a possible mass balance yield of 3.3 g of high purity nitrous oxide from a liter of waste brine, it could be feasible to recover nitrous oxide for use as an aerosol propellant or an oxidizer in fuel.

The catalytic life of the biocatalysts and their reuse potential are important considerations for process design that were not addressed in this initial investigation. For the perchlorate-reducing biocatalysts, robust activity was observed up to 23 days without any optimization of storage

conditions.[14] It is likely that chlorite dismutase would be the limiting component, since it is subject to catalytic inactivation.[35] However, recent efforts to identify an optimal chlorite dismutase for water treatment application already yielded a biocatalyst with a catalytic life up to six times greater than the one used in this study.[35] To our knowledge, no corresponding information is available on the nitrate-reducing biocatalysts, but the structural similarities between perchlorate reductase and nitrate reductase[25] suggest that at least the first step will be similarly long-lived. A variety of options are available to retain or recover biocatalysts, based on previous applications in industrial syntheses, but additional research is needed to identify the options most appropriate for these particular biocatalysts and their large-scale applications in water treatment.

5. Conclusions

To our knowledge, this is the first investigation of biocatalysts to treat real-world waste brine; the long-term goal is to improve the sustainability of ion-exchange processes. This is also the first demonstration of nitrate-reducing biocatalysts for water treatment. Biocatalytic treatment of waste brines would serve two purposes: it would prevent reintroduction of the contaminants into the environment and it would allow reuse of the brine. The impact of the first of these is difficult to quantify, as there is little information currently available on the contributions of these brines to nitrate and perchlorate in the environment. For the second, some estimates are possible. Regeneration of ion-exchange resins with a 6% sodium chloride solution represented 10.9% of the median cost and 44.8% of the median global warming potential of ion-exchange treatment,[16] suggesting the potential for substantial benefits. Application of whole-cell brine regeneration for perchlorate and nitrate at the pilot scale resulted in estimated cost reductions of 18.4%.[3] With effective removal of nitrate and perchlorate, brine reuse could be

achieved. Limitations on the number of reuse cycles would likely depend on other constituents of the source water, such as radiohalides.[3]

Biocatalysis is a new approach for environmental engineering, and further development is required to make it economically viable, particularly in the areas of biocatalyst reuse and supply of electron donors.[16] However, as brine regeneration occurs in a significantly smaller volume than drinking water treatment and is not feeding directly into distribution systems and subsequent human consumption, brine regeneration appears to be both a good potential application for biocatalytic treatment and a route to promote the technology development that could form the basis for a wide range of biocatalytic treatment processes.

Conflicts of Interest

There are no conflicts of interest to declare.

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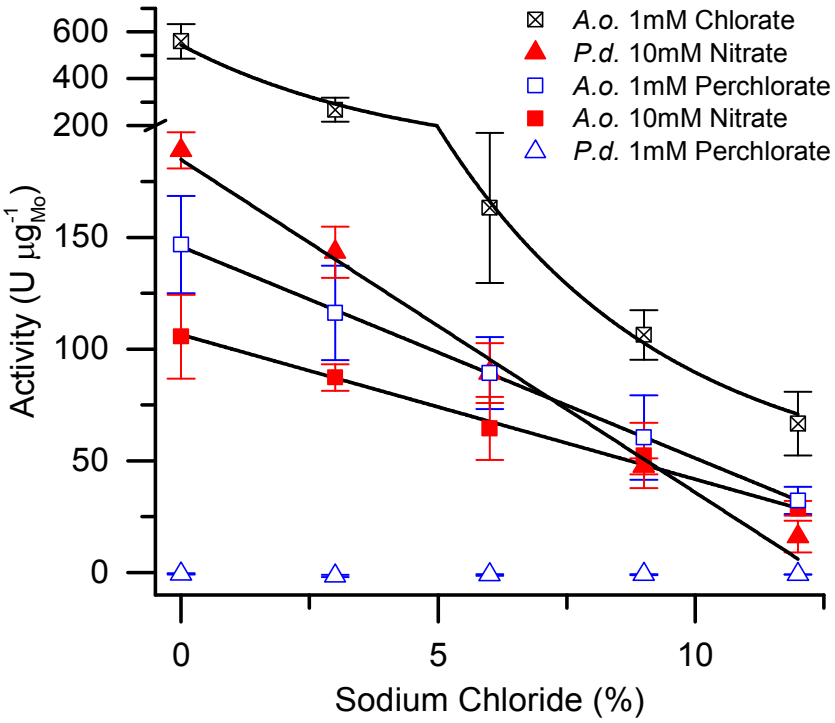
530 **Figure and Legends**

Table 1
Waste brine composition.

Component	Units	Brine[a]	% NaCl eq
pH		7.04	
Alkalinity	mM	122	
Calcium	mM	12.8	
Iron	μM	0.04	
Potassium	mM	26.9	
Magnesium	mM	15.3	
Manganese	μM	0.2	
Molybdenum	mM	0.7	
Sodium	mM	2,000	11.7% NaCl
Phosphorus	μM	12.9	
Sulfur	mM	11.3	
Ammonia	μM	<18.3	
Fluoride	mM	3.8	
Chloride	mM	2,100	12.3% NaCl
Bromide	μM	250	
Iodide	μM	23.6	
Perchlorate	μM	ND[b]	
Chlorate	μM	ND	
Nitrate	mM	151	
Bromate	mM	ND	
Iodate	mM	ND	
Sulfate	mM	25.5	
COD	ppm	407	

[a] Detection Limits – Nitrate (0.16 mM), Chlorate (95.8 μM), Perchlorate (0.5 μM), Iodate (0.5 mM), Bromate (0.4 mM), Sulfate (70.8 μM)
[b] ND – Not Detected



531

532 Figure 1: Biocatalytic activity of *A. oryzae* SPF (squares) and *P. denitrificans* SPF (triangles)
533 with perchlorate, nitrate, and chlorate. Results of MV activity assays conducted in increasing
534 sodium chloride concentration (0-12% sodium chloride). Activity is given in Units (U), defined
535 as 1 µmol MV oxidized per minute, and normalized to µg of molybdenum, an indirect measure
536 of perchlorate reductase or nitrate reductase. Double slash on y axis indicates change in scale.
537 Solid line indicates regression fit from data. Error bars are standard deviation from triplicate
538 biological replicates.

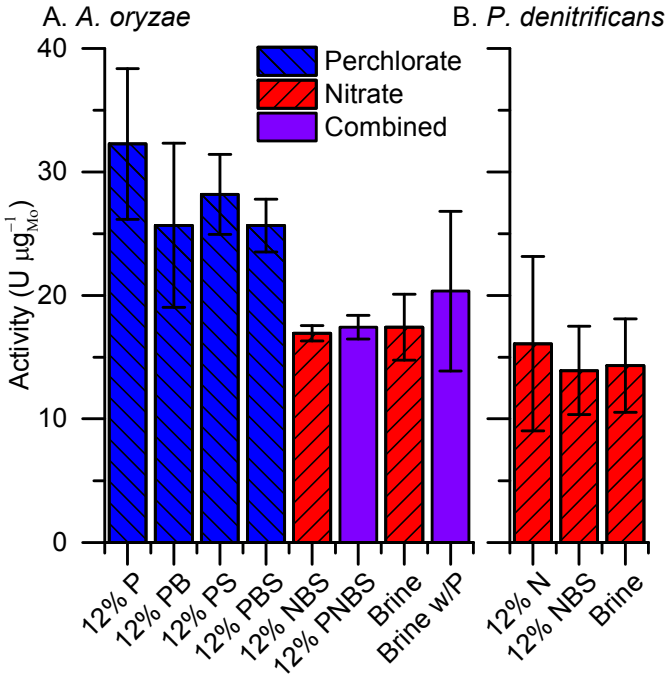
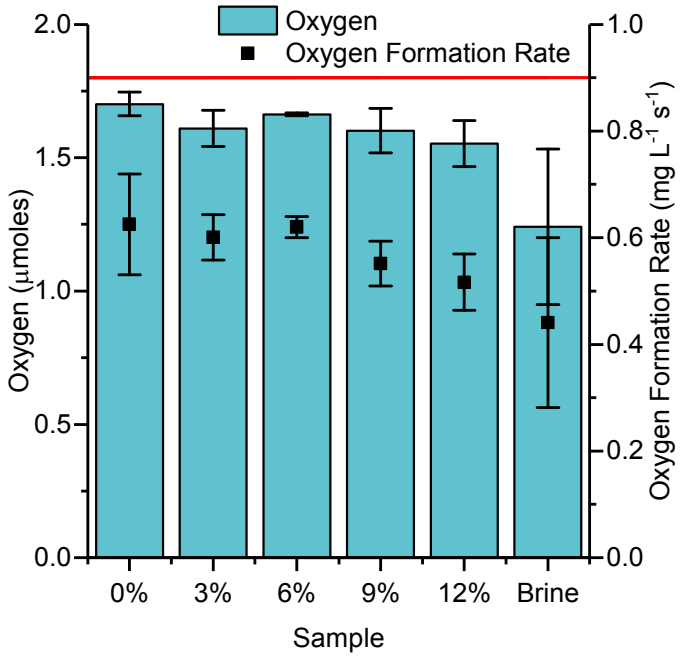
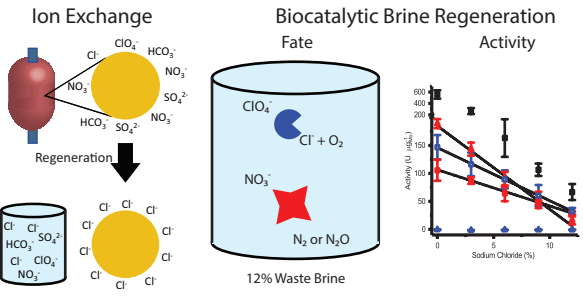


Figure 2: Biocatalytic activity of *A. oryzae* SPF (panel A) and *P. denitrificans* SPF (Panel B) in synthetic (12% sodium chloride) and waste brine for perchlorate, nitrate, and perchlorate and nitrate. Results of MV activity assays conducted with perchlorate (P) at 1mM and concentrations mimicking the waste brine: 122 mM bicarbonate (B), 25.5 mM sulfate (S), and 151 mM nitrate (N). The exception is the N only experiment, which had 10mM nitrate. Activity is given in Units (U), defined as 1 µmol MV oxidized per minute, and normalized to µg of molybdenum, an indirect measure of perchlorate reductase and nitrate reductase. Error bars are standard deviation from triplicate biological replicates.



549

550 Figure 3: Biocatalytic formation of oxygen from chlorite using *A. oryzae* SPF in synthetic
551 and waste brines. Total oxygen formed (bars) is reported in µmoles, with the red line indicating
552 the theoretical maximum determined from chlorite added (1.8 µmoles). Oxygen formation rate
553 (squares) from decomposition of chlorite is given in mg O_2 L^{-1} s^{-1}. Error bars are standard
554 deviation from triplicate biological replicates.



This work demonstrates biocatalytic reduction of perchlorate and nitrate in ion-exchange waste brines. Biocatalysis could allow regeneration of the brines and prevent reintroduction of these contaminants into the environment.